

Quality attributes and shelf life of goat's milk kefir

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INDEX

INDEX	2
List of Tables	6
List of Figures	7
List of Abbreviations	8
Abstract	9
I. Introduction	11
II. Literature Review	12
1. Microbial composition of kefir.....	12
2. Chemical composition of kefir.....	12
3. Nutritional composition of kefir.....	13
4. Types of kefir.....	13
4.1. Dairy kefir.....	13
4.2. Non-dairy kefir.....	13
5. Biological activities of kefir.....	14
5.1. Anti-hypertensive properties.....	14
5.2. Anti-carcinogenic properties.....	14
5.3. Anti-diabetic properties.....	14
5.4. Antimicrobial properties.....	15
5.5. Anti-Inflammatory properties.....	15
5.6. Antioxidant properties.....	15
5.7. Hypocholesterolemic effects.....	15
6. Production of kefir.....	15
7. Physicochemical, microbiological, and sensory characteristics of goat's milk kefir.....	17
7.1. Physicochemical characteristics.....	17
7.1.1. pH value.....	17
7.1.2. Titrable acidity.....	18
7.1.3. Texture.....	18

7.1.4. Syneresis.....	18
7.1.5. Proteolysis	18
7.2. Microbiological characteristics	19
7.3. Sensory characteristics	20
8. The storage effect on kefir.....	20
8.1. Effect on the physicochemical composition.....	20
8.1.1. pH.....	20
8.1.2. Total titratable acidities	21
8.1.3. Dry matter.....	21
8.1.4. Free amino acids.....	21
8.1.5. Total soluble solids (TSS)	22
8.2. Effect on the microbiological composition	22
9. Extending the shelf life of kefir.....	22
10. Kefir fortification	23
10.1. Plant extracts for kefir enrichment	24
10.2. Kefir fortification with juices and honey.....	25
10.3. Kefir fortification with Agro-Food waste extracts	26
10.4. Kefir enriched with encapsulated volatile oils	29
10.4.1. Protection and gradual release of bioactive compounds	29
10.4.2. Antimicrobial activity enhancement.....	29
10.4.3. Enhanced chemical composition	29
III. Materials and Methods	31
1. Kefir production	31
1.1. Grain activation	32
1.2. Kefir preparation	33
1.3. Incubation and filtration	34
1.4. Storage.....	35
2. Monitoring of shelf life of control and flavored kefir	35
2.1. Physicochemical analysis	35

2.1.1. Hydrogen potential (pH).....	36
2.1.2. Titratable acidity (TA).....	36
2.1.3. Syneresis.....	37
2.1.4. Proteolytic activity	37
2.2. Textural analysis.....	38
2.2.1. Firmness	39
2.2.2. Index of viscosity	39
2.2.3. Cohesiveness	39
2.2.4. Consistency	39
2.3. Microbiological analysis	39
2.3.1. Preparation of material for sterilization.....	39
2.3.2. Preparation of culture medium	39
2.3.3. Preparation of dilutions	40
2.3.4. Preparation of Petri dishes for LAB and MES and incubation.....	41
2.3.5. Yeasts and molds count.....	41
2.4. Sensory analysis	42
2.4.1. Preparation of the response sheet	42
2.4.2. Preparation of samples	42
3. Statistical analysis	43
IV. Results and Discussion	44
Part 1: Establishing Optimal Fermentation Conditions	44
1. Physicochemical properties	44
1.1. pH.....	46
1.2. Acidity	46
1.3. Syneresis.....	47
2. Textural properties	48
2.1. Firmness	51
2.2. Cohesiveness	51
2.3. Viscosity index	52

2.4.	Consistency	53
3.	Microbiological properties	54
3.1.	Lactic acid bacteria.....	56
3.2.	Mesophiles.....	57
3.3.	Yeasts and molds.....	58
Part 2: Assessing and Enhancing the Shelf-Life of Kefir.....		60
4.	Physicochemical properties	61
4.1.	pH.....	62
4.2.	Acidity.....	63
4.3.	Syneresis.....	67
4.4.	Proteolysis	68
5.	Textural properties	69
5.1.	Firmness	71
5.2.	Consistency	73
5.3.	Cohesiveness	74
5.4.	Index of viscosity	76
6.	Microbiological properties	78
6.1.	Lactic acid bacteria.....	80
6.2.	Mesophiles.....	82
6.3.	Yeasts	83
6.4.	Molds.....	86
7.	Sensory properties	87
V.	Conclusion Remarks and Future Perspective.....	92
	References	94

List of Tables

<i>Table 1: Chemical composition (mean) of kefir from goat's milk.....</i>	<i>18</i>
<i>Table 2: Microbial properties of kefir samples.....</i>	<i>22</i>
<i>Table 3: The fortification effect of kefir with different extracts</i>	<i>26</i>
<i>Table 4: The Effect of kefir fortification with Agro-Food waste extracts</i>	<i>27</i>
<i>Table 5: The different parameters of the two types of kefir</i>	<i>33</i>
<i>Table 6: Factors and their different values.....</i>	<i>34</i>
<i>Table 7: Experimental Design.....</i>	<i>34</i>
<i>Table 8: Analysis planning.....</i>	<i>35</i>
<i>Table 9: Analysis of variance of the response surface models fitted to the physicochemical parameters.....</i>	<i>45</i>
<i>Table 10: Summary statistics of the syneresis of kefir treatments</i>	<i>48</i>
<i>Table 11: Analysis of variance of the response surface models fitted to the textural parameters.....</i>	<i>50</i>
<i>Table 12: Analysis of variance of the response surface models fitted to the microbiological parameters.....</i>	<i>55</i>
<i>Table 13: Summary statistics of the microbiological characteristics (log CFU/ml) of kefir treatments</i>	<i>60</i>
<i>Table 14: Analyses of variance and least square means for the evolution of pH and ACIDITY in kefir</i>	<i>66</i>
<i>Table 15: Analyses of variance and least square means for the evolution of SYNERESIS and PROTEOLYSIS in kefir</i>	<i>70</i>
<i>Table 16: Analyses of variance and least square means for the evolution of FIRMNESS and CONSISTENCY in kefir.....</i>	<i>75</i>
<i>Table 17: Analyses of variance and least square means for the evolution of COHESIVENESS and VISOCITY INDEX in kefir.....</i>	<i>79</i>
<i>Table 18: Analyses of variance and least square means for the evolution of MESOPHILES and LACTIC ACID BACTERIA in kefir</i>	<i>84</i>
<i>Table 19: Analyses of variance and least square means for the evolution of YEASTS and MOLDS in kefir</i>	<i>88</i>
<i>Table 20: Descriptive statistics and analysis of variances of the sensory attributes of kefir ..</i>	<i>90</i>

List of Figures

<i>Figure 1: General process of kefir production</i>	16
<i>Figure 2: Goat's milk kefir production diagram.....</i>	33
<i>Figure 3: Response surface of pH as a function of temperature and grains/milk ratio</i>	46
<i>Figure 4: Response surface of acidity as a function of temperature and time of incubation ..</i>	47
<i>Figure 5: Response surface of syneresis</i>	47
<i>Figure 6: Response surface of firmness as a function of time and grains/milk ratio</i>	51
<i>Figure 7: Response surface of cohesiveness as a function of time and grains/milk ratio</i>	52
<i>Figure 8: Response surface of viscosity as a function of time and grains/milk ratio</i>	53
<i>Figure 9: Response surface of consistency as a function of time and grains/milk ratio.....</i>	54
<i>Figure 10: Response surface of lactic acid bacteria.....</i>	57
<i>Figure 11: Response surface of mesophiles</i>	58
<i>Figure 12: Evolution of kefir pH during shelf life</i>	63
<i>Figure 13: Evolution of kefir acidity during shelf life</i>	65
<i>Figure 14: Evolution of kefir syneresis during shelf life.....</i>	68
<i>Figure 15: Evolution of kefir proteolysis during shelf life.....</i>	69
<i>Figure 16: Evolution of kefir firmness during shelf life.....</i>	72
<i>Figure 17: Evolution of kefir consistency during shelf life.....</i>	74
<i>Figure 18: Evolution of kefir cohesiveness during shelf life.....</i>	77
<i>Figure 19: Evolution of kefir viscosity index during shelf life.....</i>	78
<i>Figure 20: Evolution of kefir lactic acid bacteria during shelf life</i>	82
<i>Figure 21: Evolution of kefir mesophiles during shelf life.....</i>	83
<i>Figure 22: Evolution of kefir yeasts during shelf life.....</i>	86
<i>Figure 23: Evolution of kefir molds during shelf life.....</i>	87
<i>Figure 24: Spider web plot of the respondents' appreciation of kefir</i>	91

List of Abbreviations

ANOVA: Analysis of Variance

BPW: Buffer Peptone Water

CIMO: Centro de Investigação de Montanha

g.s: grams per second

g.L: grams per liter

h : hour

IPB : Instituto Politécnico de Bragança

mL : millilitre

mm: millimeter

MES: Mesophiles

nm: nanometer

pH: potential of Hydrogen

rpm: rotation per minute

sd: standard deviation

UFC: Colony Forming Units

v: volume

w: weight

°D: Degree Dornic

Abstract

For centuries, traditional fermented foods have been highly valued for their unique organoleptic properties and relatively long shelf life. They are produced from various sources, particularly milk and meat. Kefir is an example of a beneficial fermented food, known worldwide for its importance in human nutrition. Kefir is a viscous, foamy liquid that resembles thick cream, characterized by its slightly gaseous, sour taste and low alcohol content. It is obtained through the fermentation of goat's, cow's, or sheep's milk using kefir grains. These grains are a symbiotic culture of yeasts and bacteria encased in a matrix of polysaccharides.

This study focuses on the processing of plain and flavored kefir made from goat's milk and their shelf life, as determined by the evolution of their microbiological, textural, and technological quality attributes. The aim of this work is to (1) determine the shelf life of goat's milk kefir by monitoring the evolution of its physicochemical, textural, and microbiological properties, and (2) assess a bio-preservation method for extending its shelf life.

All work was carried out in the Laboratory of Bacteriology of CIMO Research Centre at the Polytechnic Institute of Bragança (IPB). The research involved the production of plain goat's milk kefir through several steps, including weighing, incubating kefir grains in goat's milk, and filtering the mixture. The samples were then stored at 4°C. A full characterization of the control kefir was conducted using various physicochemical, textural, and microbiological tests at specific storage intervals to determine the optimum combination of percentage, time, and temperature.

Subsequently, plain (Control) and flavored (Treatment) goat's milk kefir were prepared. The shelf life of kefir was evaluated using statistical modeling at appropriate storage intervals for the different analyses. Sensory analysis was also conducted on control and flavored kefir samples. Finally, the data collected was modelled.

This study has demonstrated the unequal influence of several factors—milk type, kefir grains, temperature, incubation time, grains/milk ratio, and kefir aromatization—on the final quality attributes of kefir. Consequently, kefir production must be highly selective in terms of milk and grain types, while also implementing strict controls on incubation parameters throughout the manufacturing process. Adherence to necessary hygiene practices and compliance with national and international food standards are essential. Additionally, consumers should be informed of kefir's final composition to avoid any risk of intolerance.

Resumo

Durante séculos, os alimentos fermentados tradicionais foram altamente valorizados por suas propriedades organolépticas únicas e pelo seu prazo de validade relativamente longo. Esses alimentos são produzidos a partir de diferentes fontes, principalmente leite e carne. O kefir é um exemplo de alimento fermentado benéfico, conhecido mundialmente por sua importância na nutrição humana. Trata-se de um líquido viscoso e espumoso que se assemelha a um creme espesso, caracterizado pelo seu sabor azedo ligeiramente gasoso e baixo teor alcoólico. O kefir é obtido pela fermentação do leite de cabra, vaca ou ovelha, utilizando grãos de kefir, que são formados por uma simbiose de leveduras e bactérias envoltas em uma matriz de polissacarídeos.

Este estudo foca-se no processamento de kefir simples e aromatizado de leite de cabra e no seu tempo de conservação, determinado pela evolução de seus atributos de qualidade microbiológica, textural e tecnológica. Os objetivos deste trabalho são: (1) determinar o tempo de vida útil do kefir de leite de cabra, monitorando a evolução de suas propriedades físico-químicas, texturais e microbiológicas, e (2) avaliar um método de bioconservação para prolongar sua vida útil.

Todo o trabalho foi realizado no laboratório do Centro de Investigação de Montanha (CIMO) do Instituto Politécnico de Bragança (IPB). O estudo envolveu a produção de kefir simples de leite de cabra por meio de uma série de etapas, incluindo a pesagem, incubação dos grãos de kefir no leite de cabra e filtragem. As amostras foram então armazenadas a 4°C. Foi realizada uma caracterização completa do kefir de controle, utilizando diversos testes físico-químicos, texturais e microbiológicos em intervalos específicos de armazenamento para determinar a combinação ideal de proporção, tempo e temperatura.

Em seguida, foram preparados kefir de leite de cabra simples (controle) e aromatizado (tratamento). O prazo de validade do kefir foi avaliado com base em modelagem estatística, aplicando-se intervalos de armazenamento apropriados para cada análise. A análise sensorial também foi realizada nas amostras de kefir simples e aromatizado. Por fim, os dados coletados foram modelizados.

Este trabalho demonstrou que os atributos finais de qualidade do kefir dependem de vários parâmetros de forma desigual, como o tipo de leite e de grãos, a temperatura, o tempo de incubação, a proporção de grãos/leite e a aromatização. Consequentemente, a produção de kefir deve ser altamente seletiva em relação ao tipo de leite e de grãos, além de seguir rigorosamente os parâmetros de incubação durante o processo de fabricação, aplicando as práticas de higiene necessárias e cumprindo as normas alimentares nacionais e internacionais. Ademais, é essencial que os consumidores sejam informados sobre a composição final do kefir, para evitar quaisquer riscos de intolerância.

I. Introduction

Probiotics are live microorganisms administered in sufficient quantities to confer health benefits (FAO/WHO, 2001). They offer numerous benefits for human health, including anti-carcinogenic and anti-mutagenic activities. Probiotics are also used in the treatment of inflammatory bowel disease, combating infection by *Helicobacter pylori*, and preventing gastrointestinal disorders. Kefir has also been proven to strengthen the immune system, reduce lactose intolerance and blood cholesterol levels, and provide relief in the treatment of colds and flu (Cruz et al. 2010).

In recent years, the use of probiotic foods has increased worldwide. In Europe, the probiotic food market has reached a total value of 1.4 billion euros, driven primarily by probiotic yogurts and desserts, which account for around 72% of the total market (Cruz et al. 2010). Kefir is one of the most popular probiotic drinks. It is a fermented, acidic, and alcoholic milk product, typically made from cow's, sheep's, or goat's milk, as well as plant-based sources such as soy, rice, and coconut. It is unique among fermented milk products because it undergoes both lactic acid and alcoholic fermentation. The drink originated in the South Caucasus, where it is still prepared today under various names. The most common name, "kefir," is of Turkish origin (Prado et al. 2015).

Goat's milk has higher protein, non-protein nitrogen, and phosphate levels compared to cow's milk. It also has a greater buffering capacity. Additionally, goat's milk contains smaller fat globules, a higher percentage of short- and medium-chain fatty acids, and forms a softer curd, which contributes to better digestibility and healthier lipid metabolism (Dewi et al. 2020).

It is important to note that the physicochemical and microbial quality of kefir made from fermented milk is influenced by several factors, including the type of milk, the grain/ milk ratio, fermentation time and temperature, and storage conditions. Monitoring the hygienic and sensory quality of these products is also crucial.

In this context, the present study aims to understand the evolution of microbiological, textural, and technological quality attributes to determine the shelf life of plain goat's milk kefir.

Additionally, this research will explore a natural preservative with the potential to extend the shelf life of kefir. The objectives of this study were (1) to determine the optimal parameter of incubation time, temperature time and grain/milk ratio for the production of goat's milk kefir of good technological and microbiological properties; and (2) to monitor the evolution of the physicochemical, textural, and microbiological properties of goat's milk kefir to assess its deterioration in time.

II. Literature Review

Kefir is a health-promoting fermented dairy beverage that contains a diverse array of bacterial and fungal microorganisms. It is a viscous, acidic, and mildly alcoholic product produced through the fermentation of milk using a mixed microbial population, which is confined to “kefir grains” as the starter culture (FAO/WHO, 2011). The starter culture, derived from kefir grains, consists of *Lactobacillus kefir*, along with species from the genera *Leuconostoc*, *Lactococcus*, and *Acetobacter*, which grow in a highly specific symbiotic relationship. Kefir grains also contain both lactose-fermenting yeasts, such as *Kluyveromyces marxianus*, and non-lactose-fermenting yeasts, including *Saccharomyces unisporus*, *Saccharomyces cerevisiae*, and *Saccharomyces exiguus* (CODEX ALIMENTARIUS, 2022).

Kefir is characterized by gelatinous, irregularly shaped grains formed by a consortium of yeasts and lactic acid bacteria. This combination induces both acid-alcoholic fermentation in sugar and milk solutions (Schneedorf and Anfiteatro, 2004).

1. Microbial composition of kefir

Kefir consists of 65 to 90% bacteria from the *Lactobacillaceae* family, including members of the genera *Lactococcus*, *Streptococcus*, *Leuconostoc*, and *Acetobacter*, with the remainder being yeasts. The predominant species include *Lactocaseibacillus paracasei*, *Lactobacillus acidophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lactiplantibacillus plantarum*, *Lactobacillus helveticus*, *Leuconostoc mesenteroides*, *Lactococcus lactis*, *Lentilactobacillus kefir*, *Lentilactobacillus parakefir*, and *Lactobacillus kefir* (González-Orozco et al., 2022).

2. Chemical composition of kefir

Kefir grains have a variable chemical composition. Samples of kefir grains from Russia, Sweden, Yugoslavia, and Bulgaria are composed of approximately 90% water, 2-3% proteins, 0-3% lipids, 5-8% soluble non-nitrogenous substances, and 0-7% ash. In contrast, kefir grains from Argentina contained 83% water, 9-10% polysaccharides, and 4-5% proteins (Garrote, Abraham, and De Antoni 2001). Moisture is the predominant constituent (90%), followed by sugars (6%), fats (3-5%), proteins (3%), and ash (0-7%) (Prado et al. 2015). According to this author, kefir consists of 4.4% fat, 12.1% ash, 45.7% mucopolysaccharides, 34.3% total proteins (27% insoluble proteins, 1.6% soluble proteins, and 5.6% free amino acids), vitamins B and K, tryptophan, and minerals such as Ca, P, and Mg. Shaped similarly to cauliflower, kefir grains are elastic, irregular, gelatinous, ivory or white, and vary in size from 0.3 to 3-5 cm in diameter.

3. Nutritional composition of kefir

Kefir has a variable nutritional composition that depends on several factors, including the milk used, the origin and constitution of the kefir grains, as well as the duration and temperature of fermentation and storage. During fermentation, proteins become more easily digestible due to acid coagulation and proteolysis. Kefir is rich in amino acids such as serine, lysine, alanine, threonine, tryptophan, valine, methionine, phenylalanine, and isoleucine. According to Prado et al. (2015), the following amino acids were quantified in plain kefir: lysine (376 mg/100 g), isoleucine (262 mg/100 g), phenylalanine (231 mg/100 g), valine (220 mg/100 g), threonine (183 mg/100 g), methionine (137 mg/100 g), and tryptophan (70 mg/100 g).

4. Types of kefir

Kefir varies based on the diverse microbial constituents found within the kefir grains, resulting in different kefir products with distinct microbiological, physicochemical, nutritional, and sensory characteristics.

4.1. Dairy kefir

Kefir is an artisanal dairy drink produced through the fermentation of milk sugar (lactose) by bacteria and yeast from kefir grains. The production process involves adding kefir grains to pasteurized milk cooled to 20-25°C, where fermentation begins with the inoculation of the kefir grains, which serve as natural ferments. The raw milk used to produce kefir must be low in bacteria, somatic cells, and free from pathogens or unwanted substances such as antibiotics and disinfectant residues. This lactic yeast-fermented beverage has a viscous, slightly effervescent texture and a low alcohol content, ranging from 0.08% to 2.0% (Azizi et al., 2021).

4.2. Non-dairy kefir

There are also non-dairy kefir products such as water kefir, sweet kefir, or tibico, which gained significant popularity during the 20th century due to their associated health benefits. These products are typically produced by fermenting kefir grains in a sugar solution (brown sugar) but can also be prepared with alternative substrates such as fruit juices (e.g., apple, pineapple, grape, quince, kiwi, pear, pomegranate, melon, strawberry, tomato, coconut), vegetables (e.g., ginger, onion, soy, fennel, carrot), and molasses (e.g., sugar cane, honey). This makes kefir accessible to people with lactose intolerance and those following a vegan lifestyle. Indeed, sugar water or fruit juices are rich in water, carbohydrates, and nutrients like proteins, amino acids, vitamins, and minerals, creating a favorable environment for microbial growth. This environment promotes a rapid increase in the biomass of kefir grains, which are used to prepare fermented drinks such as kefir.

Non-dairy kefir is produced using kefir grains, which are composed of a consortium of yeasts, primarily *Kluyveromyces*, *Candida*, and *Saccharomyces*, along with lactic acid bacteria (LAB) such as *Lactobacillus*, *Lactococcus*, *Leuconostoc*, and *Streptococcus*. These microorganisms are integrated into a natural exopolysaccharide (EPS) matrix. The production process involves directly adding kefir grains to a pasteurized and cooled substrate (e.g., fruit juice, sugar solution) and incubating the mixture for approximately 24 hours at 25-30 °C. After fermentation, the grains are separated from the kefir by sieving, followed by washing, drying at room temperature, and storing in a cooling tank for future fermentation use (Azizi et al., 2021).

5. Biological activities of kefir

Throughout history, kefir has been recommended as a remedy for various serious illnesses, including tuberculosis, cancer, and gastrointestinal disorders. Recently, numerous studies on kefir's bioactivities as a natural drink have uncovered various health benefits, which can be attributed both to the presence of probiotic microorganisms and the wide range of bioactive compounds produced during the fermentation process (Azizi et al., 2021).

5.1. Anti-hypertensive properties

High blood pressure can lead to serious health issues, including heart attacks, strokes, and other cardiovascular diseases. Scientific studies have proven that kefir is a beneficial health food. The symbiotic metabolic interactions between certain species of bacteria and yeasts in kefir, which facilitate the proteolytic and lipolytic breakdown of milk compounds, contribute to various positive health effects, including antihypertensive properties effects (Azizi et al., 2021).

5.2. Anti-carcinogenic properties

Although genetic factors play a major role in cancer risk, it has been proven that certain lifestyle choices can significantly reduce the likelihood of developing cancer. Kefir's anti-carcinogenic effects are demonstrated through its ability to slow tumor growth by promoting apoptosis, enhancing immune response, modulating the intestinal microbiota, reducing tumor size and DNA damage, facilitating antioxidative processes, inhibiting cell proliferation, and deactivating pro-carcinogenic agents (Azizi et al., 2021).

5.3. Anti-diabetic properties

Kefir has a positive effect on blood sugar control. Studies have shown that kefir can serve as an effective complementary therapy for the treatment and prevention of diabetes and is also recommended as a nutritional approach for glycemic management. Specifically, kefir can help reduce insulin and fasting blood glucose (FBS) levels without significantly affecting HbA1c (glycated hemoglobin) levels (Salari et al., 2021).

5.4. Antimicrobial properties

The microbiota of kefir has demonstrated promising antimicrobial effects, with certain bacteria and yeasts exhibiting antimicrobial activity both in vivo and in vitro against enteropathogenic bacteria and spoilage fungi. Kefir can positively impact human health through various mechanisms of action, including the inhibition of pathogenic bacteria, protozoa, and viruses. It has been shown to possess antimicrobial activity against a wide range of bacteria and fungi, providing a protective barrier against pathogens, specifically offering resistance to enterohemorrhagic *Escherichia coli*, *Helicobacter pylori*, and *Staphylococcus aureus* (Rodrigues, Carvalho, and Schneedorf, 2005). In some instances, kefir has shown antimicrobial potency comparable to that of conventional antibiotics in vitro. The antimicrobial actions of the kefir microbiota include modulation of the immune system, adhesion to the intestinal epithelium, stimulation of barrier defenses, inactivation of toxins, competition for nutrients and adhesion sites, and the secretion of antimicrobial substances (González-Orozco et al. 2022).

5.5. Anti-Inflammatory properties

The consumption of kefir has been shown to exert immunomodulatory effects, strengthen pathogenic barriers, and provide anti-neoplastic and digestive benefits. Additionally, kefir exhibits significant antimutagenic and antioxidant activities, as demonstrated in *Salmonella* mutagenicity and radical scavenging assays.

5.6. Antioxidant properties

Although the human body possesses an intrinsic antioxidant system, the consumption of natural antioxidants through food is essential. These antioxidants help protect the body from free radical damage and can delay the progression of many chronic diseases. Milk kefir, in particular, has been shown to exhibit significantly higher scavenging activity against DPPH radicals, inhibition of linoleic acid peroxidation, and notable reducing power (Liu, Chen, and Lin, 2005).

5.7. Hypocholesterolemic effects

Kefir can be considered a promising food for preventing cardiovascular diseases due to its hypocholesterolemic effects. A study conducted on hamsters demonstrated a significant decrease in serum triacylglycerol and total cholesterol levels, along with reduced cholesterol accumulation in the liver (Liu et al., 2006).

6. Production of kefir

The production of kefir is distinctive and subject to quality variations due to the fluctuating microbiological composition of the kefir grains used during fermentation (Zourari and Anifantakis, 1988). The process steps involved in kefir production are illustrated in Fig. 1.

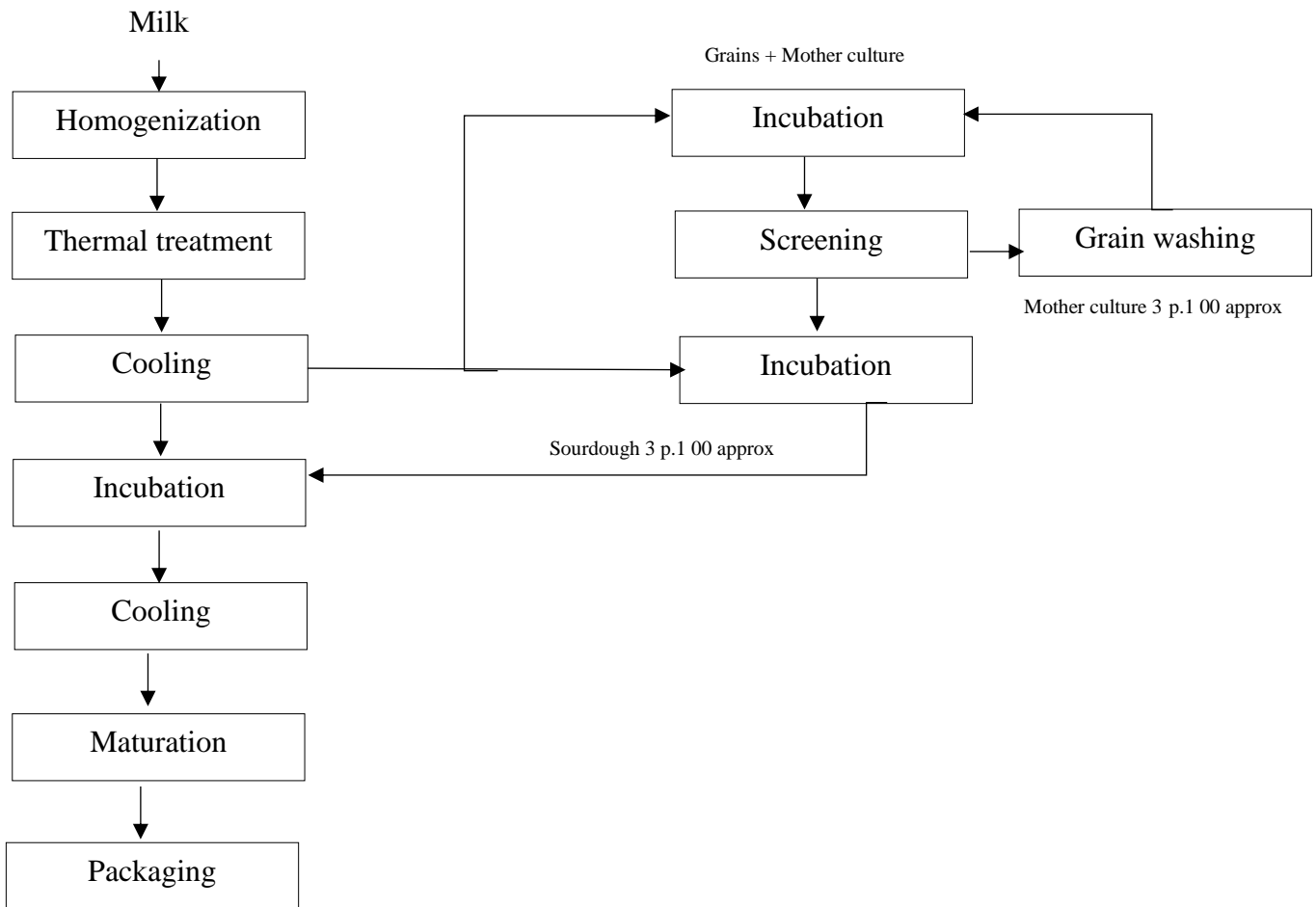


Figure 1: General process of kefir production

Preservation of kefir grains: In their fresh state (stored cold at 4°C), kefir grains become inactive within 8 to 10 days when kept in sterile water or whey. When dried at room temperature (for 36 to 48 hours) and subsequently stored cold, they can be fully reactivated after 2 to 3 cycles of re-culturing in milk, even after a year or a brief storage period. Freeze-drying and freezing are the best methods for preserving kefir grains over extended periods.

Milk homogenization: To achieve a better texture in the finished kefir product, it is essential to homogenize the milk under high pressure and preheat it to the homogenization temperature (e.g., 15 MPa and 70°C).

Heat treatment of milk: Heat treatment is carried out at high temperatures for extended durations (e.g., 90-95°C for 2 to 30 minutes or 85-87°C for 5 minutes to 1 hour) to ensure significant water retention, which reduces syneresis and increases viscosity. Additionally, it eliminates the majority of microbial flora and facilitates the utilization of milk proteins by the fermentation microflora.

Milk cooling and incubation: Once the pH of the curd reaches 4.5 to 4.6, it is cooled to 4-6°C within a maximum of 20 minutes to prevent further pH reduction. Incorporating air into the curd should be avoided, as it increases the risk of product stratification.

Curd cooling: The product is cooled until 4-6°C for a maximum of 20 minutes when the pH is between 4.5 to 4.6, the aim of which is to prevent the pH from further decreasing. The incorporation of air into the curd increases the risk of stratification of the product; therefore, this must be avoided.

Maturation and packaging: After cooling, the product is transferred to a stabilization tank where denatured proteins absorb maximum water. The curd is gently separated to preserve its structure and then stored in a cold room (8°C to 16°C) for 12 hours to 6 days for maturation. At the end of this stage, the pH should reach 4.3 to 4.4 (Zourari and Anifantakis, 1988).

7. Physicochemical, microbiological, and sensory characteristics of goat's milk kefir

7.1. Physicochemical characteristics

The physicochemical characterization of goat's milk kefir involves the determination of pH, titratable acidity, texture, syneresis, and proteolysis. These parameters play a crucial role in the overall acceptability of the product. As shown in Table 1, the content of minerals and organic materials (fats, proteins, and carbohydrates) varies between samples, influenced by the composition and activity of the microorganisms. These microbial interactions can affect the organoleptic quality of the kefir during consumption.

7.1.1. pH value

The pH, or hydrogen potential, is a measure of the acidity or alkalinity of a solution. It is defined by the concentration of hydronium ions (H_3O^+), monitoring the pH allows to control the fermentation process and obtain a kefir that is safe to consume, with the desired organoleptic properties. A final pH between 4.0 and 4.6 is generally considered optimal for kefir. A pH below 4.6 promotes the safety of kefir by inhibiting the proliferation of pathogens and contributing to its natural preservation thanks to its antimicrobial properties. In addition, this level of acidity positively influences the growth and activity of the beneficial lactic acid bacteria present in kefir.

Table 1: Chemical composition (mean) of kefir from goat's milk

pH	Syneresis (%)	Total solid (%)	Fat (%)	Protein (%)	Ash (%)	Carbohydrate (%)	Reference
4,30	23,58	12,16	3,04	3,08	0,56	5,49	(Akan,2020)
4.50	-	-	3.51	-	-	-	(Irigoyen, 2005)
5,69	-	-	2,82	-	-	-	(Dewi et al. 2020)
4,30	8,86	-	-	-	-	-	(Ozcan, Yilmaz-Ersan, Akpinar Bayizit, et al. 2018)
4.34	-	-	-	-	-	-	(Putri et al., 2020)
4.47	-	-	3.18	3.06	-	-	(Sarica and Coşkun 2020)
4,54	-	-	-	3.4	-	4.4	(Torre et al. 2024)
-	-	-	1,28	3,2	0.58	4,9	(Alves et al. 2021)

7.1.2. Titrable acidity

Titration acidity is an approximation of total acidity, measuring both associated and dissociated hydrogen ions. It quantifies the amount of a strong base (NaOH) required to reach a basic pH, is determined in the presence of phenolphthalein and is expressed in Dornic degrees (Sulmiyati et al., 2019).

7.1.3. Texture

Texture is measured using a texturometer to determine the product's physical characteristics, such as hardness, fracture resistance, adhesiveness, gel strength, and extensibility.

7.1.4. Syneresis

According to Schmidt and Bouma (1992), syneresis is measured by sampling kefir after degreasing through centrifugation and filtration. It is expressed as the percentage of free whey. Refer to Table 2 for syneresis values.

7.1.5. Proteolysis

Proteolysis is the hydrolysis of peptide bonds, breaking down proteins into smaller peptides and/or individual amino acid residues. This process is catalyzed by chemicals or enzymes to accelerate the reaction (Shantha Raju, 2019). The results indicate that two samples exhibited a syneresis rate of $20.66 \pm 3.09\%$ and $20.90 \pm 1.73\%$, respectively. These values are notably lower than those recorded for other samples, suggesting that these two samples, primarily composed of kefir grains sourced from Germany, demonstrate a superior water retention capacity. This characteristic likely contributes to a more stable curd structure, enhancing the milk retention ability relative to the other samples.

Furthermore, by the end of fermentation, the pH values across samples ranged narrowly, from 4.24 ± 0.05 to 4.34 ± 0.12 , indicating a relatively consistent acidity level, which could be linked to uniform fermentation conditions across samples. This stability in pH is essential as it directly influences curd formation, microbial activity, and overall product quality. The two samples displayed flow curves positioned above those of other samples, indicating a higher flow

behavior, which aligns with a more pronounced resistance to flow at lower shear rates. This high flow behavior may be associated with larger particle sizes within these samples, as previous research has established a positive correlation between particle size and apparent viscosity in fermented dairy products (Wang and Guo, 2022). Interestingly, at a shear rate of 50 s^{-1} , the viscosity of the two samples aligns with or closely resembles that of the other samples. This suggests that under conditions that mimic the natural swallowing process, the viscosity levels converge, resulting in a consistent texture across all samples when consumed. This consistency in viscosity at higher shear rates is important, as it ensures that the mouthfeel and swallowability remain uniform.

7.2. Microbiological characteristics

The microflora of kefir, comprising a complex set of microbial groups including mesophilic streptococci, *Leuconostoc*, mesophilic or thermophilic lactobacilli, yeasts, and acetic bacteria (Zourari and Anifantakis, 1988), forms a unique microbial ecosystem that directly influences kefir's texture and flavor. In analyzing microbial species present in the samples, a study using the RDP Classifier (with a 0.7 confidence threshold) and the Green Gene Release 13.5 database (Wang and Guo, 2022) found a notable dominance of the *Lactobacillus* genus across samples, illustrating its central role in fermentation and probiotic benefits. Following *Lactobacillus*, other genera such as *Bacillus*, *Micrococcus*, and *Enterococcus* had relative abundances above 4.42%, suggesting these bacteria also contribute significantly to kefir's microbial composition and potential health benefits. Interestingly, *Acetobacter*, a genus typically found in kefir grains, was present at a lower abundance (less than 2.26%) in goat milk kefir, which marks a distinctive difference in the microbial profile of goat milk kefir compared to traditional kefir grains. This limited presence of *Acetobacter* in goat milk kefir may indicate that the bacterial composition of reconstituted kefir grains is partly constrained by the microbial characteristics of the original kefir grains. These findings emphasize that while kefir's microbial environment can be modified, the core bacterial structure remains influenced by the source kefir grains, ultimately affecting the fermentation profile and final product attributes.

The relative abundances of dominant bacteria in goat's milk kefir reveal the microbial profile where *Lactobacillus* stands out as the predominant genus, followed by *Bacillus*, *Micrococcus*, and *Enterococcus* with over 4.42% relative abundance. This bacterial composition is critical to the fermentation process and health benefits associated with goat's milk kefir. *Kazachstania* is the predominant genus among yeasts, showing the highest relative abundance in goat's milk kefir samples compared to other genera, which underscores its significant role in the fermentation and flavor development of kefir. Yeast genera are the most abundant in all kefir

samples. *Kluyveromyces* and *Saccharomyces* appear at much lower levels, each with relative abundances below 1.0%. The overall low abundance of molds (<1%) across samples is attributed to the competitive inhibition of yeast growth, enhancing both the safety and quality of the kefir. This minimal mold presence supports a safer product with a lower likelihood of spoilage, contributing to the positive quality profile of goat's milk kefir (Wang and Guo, 2022).

7.3. Sensory characteristics

The organoleptic (sensory) qualities of kefir, such as taste, texture, and aroma, are closely tied to the biochemical transformations that occur due to the microbial activity within kefir grains and the fermentation starter culture. These qualities vary depending on specific aspects of the fermentation and maturation process, including temperature, time, and microbial composition (Zourari and Anifantakis, 1988). The balance of lactic acid bacteria and yeasts, along with their metabolic byproducts, greatly influences kefir's characteristic tangy flavor, creamy texture, and effervescence.

8. The storage effect on kefir

Proper storage is crucial for maintaining kefir's quality, as temperature and storage duration significantly impact both its physicochemical characteristics and microbial profile. Monitoring these conditions helps preserve kefir's desirable qualities and extends its shelf life.

8.1. Effect on the physicochemical composition

During refrigerated storage, kefir's physicochemical properties such as pH, acidity, total dry matter, and total free amino acid content gradually change. For instance, a study by Akan (2020) showed that pH tends to decrease slightly, and acidity increases over time as microbial activity continues, albeit at a reduced rate due to cold temperatures. Total dry matter and free amino acid levels also tend to fluctuate, influencing texture and nutritional quality. Therefore, optimizing storage conditions is essential to maintain kefir's desirable physicochemical attributes and ensure a high-quality product for consumption throughout its shelf life.

8.1.1. pH

As storage time increases, kefir demonstrates a distinct decline in pH levels, indicating ongoing acidification. Specifically, kefir pH values range between 4.02 and 4.35, with a notable drop occurring between the 5th and 6th day of storage (Akan, 2020). This pH decline is attributed to ongoing microbial activity, particularly the production of organic acids by lactic acid bacteria, even under refrigeration. This gradual acidification impacts kefir's flavor profile, making it tangier over time, and can also affect texture due to changes in protein stability. Understanding this pH shift is essential for producers and consumers alike, as it informs optimal storage duration for maintaining kefir's intended taste, texture, and overall quality.

8.1.2. Total titratable acidities

The acidity of kefir levels ranged during storage between 0.77 and 0.92 g lactic acid per 100 mL (Akan, 2020). This pattern suggests that early in storage, microbial activity may stabilize or slow, resulting in a temporary reduction in acid production. However, as storage continues, certain microbial populations may resume or accelerate acid production, leading to the observed increase in acidity. These changes in acidity impact the flavor profile, contributing to a tangier taste over time, which is typical of fermented dairy products like kefir. Maintaining the balance in acidity is critical for ensuring the desired sensory qualities and shelf life of the product.

The study examined the effect of storage time on kefir over a 24-day period, with lactic acid measurements taken every 4 days. Results indicated significant variability in lactic acid content, with values ranging from 1.97% (for kefir produced through incubator fermentation and stored at room temperature for 16 days) to 3.54% (for kefir fermented at room temperature and subsequently stored under refrigeration for 24 days). These findings suggest that both fermentation conditions and storage temperature play a significant role in determining the lactic acid concentration in kefir. However, the lactic acid levels observed in some cases exceeded the recommended range set by the Indonesian National Standard (SNI) for yogurt, which applies to kefir as well, recommending a range of 0.5% to 2.0% lactic acid. Exceeding this range may lead to undesirable sensory qualities, such as an overly sour taste and potential changes in texture, highlighting the need for controlled storage and fermentation conditions to produce a kefir with optimal flavor and quality (Putri, Setiani, and Warya, 2020).

8.1.3. Dry matter

The total dry matter content in kefir starts at an initial value of 11.11 g/100 g on the 0th day, which slightly decreased to 10.41 g/100 g by the 6th day. Despite these small fluctuations, the total dry matter content did not experience significant changes over the storage period, indicating that storage time has a minimal impact on this parameter (Akan, 2020).

This stability in total dry matter content suggests that the structural components of kefir remain largely unaffected by prolonged storage, which may be beneficial for maintaining the intended texture and consistency of the product.

8.1.4. Free amino acids

The changes in free amino acids are indicative of ongoing biochemical processes, even during refrigerated storage, which can impact both the nutritional profile and safety of kefir. Regular monitoring of these compounds in stored kefir is essential to maintain optimal product quality and ensure safe consumption (Akan, 2020).

8.1.5. Total soluble solids (TSS)

A significant and progressive decline in Total Soluble Solids (TSS) content was observed in fruit kefir samples during refrigerated storage at 7°C over a 28-day period, as indicated by Brix measurements. This decline reflects a reduction in the Monosaccharide Equivalent Solids (MES) within the kefir. Specifically, TSS content decreased by 6.27% in the first sample and 5.27% in the second over the storage period (Bueno et al., 2021). This reduction in TSS suggests that sugars and other soluble solids in fruit kefir are being consumed by active microorganisms, even at low storage temperatures, which can impact the taste, sweetness, and nutrient profile of the product. Such monitoring of TSS in fruit kefir provides insight into the product's stability and sensory qualities over time, essential for managing shelf life and consumer expectations.

8.2. Effect on the microbiological composition

The microbiota of kefir comprises a symbiotic mixture primarily of *Lactobacilli*, lactic streptococci, yeasts, and acetic bacteria, with lactic acid bacteria and yeasts as the dominant species (Ozcan et al., 2018). In a study examining the microbiological composition of dairy and fruit kefir during storage, notable differences in microbial counts were observed between the two types of kefir over time, as shown in Table 2. For dairy kefir, the average count of all microbial species, particularly *Lactobacilli*, showed a marked decline throughout the storage period. Specifically, the *Lactobacilli* population dropped from an initial count of 7.60×10^8 to 2.33×10^8 CFU/mL. Similarly, fruit kefir exhibited a decrease in *Lactobacilli* from 5.08×10^8 to 4.65×10^8 CFU/mL, although the decline was less pronounced than in dairy kefir.

Table 2: Microbial properties of kefir samples (Ozcan et al., 2018)

Storage	Dairy Kefir		Fruity Kefir	
	1 st day	Last day	1 st day	Last day
<i>Lactobacilli</i>	$7,60 \times 10^8$	$2,33 \times 10^8$	$5,08 \times 10^8$	$4,65 \times 10^8$
<i>Lactococci</i>	$1,35 \times 10^9$	$2,58 \times 10^8$	$4,34 \times 10^8$	$4,94 \times 10^8$
<i>Acetic Acid Bacteria</i>	$3,31 \times 10^7$	$9,39 \times 10^6$	$9,71 \times 10^6$	$1,06 \times 10^7$
<i>Yeasts</i>	$2,53 \times 10^5$	$9,86 \times 10^4$	$1,08 \times 10^4$	$1,62 \times 10^5$

9. Extending the shelf life of kefir

Shelf life refers to the period during which kefir remains safe for consumption and free from physical or sensory (organoleptic) changes that could affect its quality. This duration represents the maximum recommended time for safe storage, consumption, and enjoyment of the product, provided it is maintained under optimal conditions for use, packaging, distribution, and storage (Solanki, Ghosh, and Kumawat, 2023). To extend kefir's shelf life effectively, key considerations include:

- **Optimal storage temperatures:** Kefir is typically stored at low temperatures (4–7°C) to slow microbial activity and acid production, which helps maintain pH stability, texture, and taste.

- **Controlled packaging:** Proper packaging (such as airtight containers) reduces oxygen exposure, limiting the growth of aerobic spoilage organisms and delaying undesirable fermentation.
- **Addition of preservatives or biopreservatives:** Natural preservatives, such as nisin, lysozyme, or probiotics with antimicrobial properties, can help inhibit spoilage organisms and extend kefir's freshness.
- **Minimizing light exposure:** Reducing light exposure prevents degradation of sensitive components in kefir, such as vitamins and some probiotic strains.
- **Regular monitoring of microbial counts and acidity:** Tracking changes in microbial populations and acidity over time ensures that kefir remains within safe and quality-assured parameters, adjusting storage recommendations as needed.

Extending kefir's shelf life while retaining its nutritional and probiotic properties is essential for consumer satisfaction, food safety, and reducing waste. These practices help balance quality maintenance with prolonged storage potential, ultimately supporting a reliable and safe product. The integration of plant-based components into fermented dairy products, like kefir, is an emerging trend in the dairy industry aimed at creating functional foods with added health benefits. This approach aligns with current research efforts to extend kefir's shelf life using natural, plant-derived additives that possess antibacterial and antioxidant properties, reducing oxidation and, thus, enhancing product stability. One promising method involves incorporating organic plant-based preservatives directly into ready-to-use kefir. For example, adding a powdered blend made from *Cladonia* lichens, lingonberries, and bearberry leaves has been shown to extend kefir's shelf life to up to 16 days without altering its taste. This powder is produced through a single-stage mechanical-chemical activation process, wherein plant-based ingredients are ground together in a ball mill at high speeds (1200-1500 rpm) for 1-2 minutes. This approach avoids the use of solvents, making it a more environmentally friendly and sustainable option. The advantages of this method are manifold: it is technically simple, cost-effective, and eco-friendly, providing an innovative way to enhance kefir's stability naturally without impacting the flavor profile of the final product. Such advancements offer a promising alternative to synthetic preservatives and align with consumer demand for clean-label, naturally preserved products in the dairy industry (Vasilevna and Konstantinovna, 2020).

10. Kefir fortification

Flavored fermented milks, such as fortified kefir, are categorized as composite milk products under the General Standard for the Use of Dairy Terms (CXS 206-1999).

According to Section 2.3 of this standard, such products can contain up to 50% (m/m) non-dairy ingredients. These ingredients, including sweeteners, fruits, vegetables, juices, purees, cereals, honey, chocolate, nuts, coffee, spices, and other natural flavorings, can be added either before or after fermentation, allowing for tailored flavor and nutritional profiles (Codex Alimentarius, 2022).

The fortification of kefir with plant and agro-food waste extracts is an effective strategy for enhancing its nutraceutical benefits. The bioactive molecules derived from these plant-based ingredients introduce health-promoting properties, such as antioxidants, vitamins, and polyphenols, which can support immune health, digestion, and metabolic function. Additionally, using extracts from agro-food waste supports sustainability by repurposing food byproducts, aligning with eco-conscious consumer preferences.

These plant-based additives also influence kefir's:

- **Sensorial properties:** The addition of plant extracts and fruit-based ingredients can alter the taste, texture, aroma, and color of kefir, potentially adding appealing flavors and enhancing its sensory profile.
- **Chemical properties:** Bioactive compounds, such as antioxidants, can help preserve the product by reducing oxidative reactions, thus contributing to a longer shelf life.
- **Microbiological properties:** Some plant-derived additives may interact with the existing kefir microbiota, possibly affecting fermentation rates and microbial balance, which could influence texture, acidity, and probiotic content. Evaluating these effects is essential for producing a fortified kefir that not only meets consumer demand for health-enhancing ingredients but also retains high quality in taste, safety, and probiotic efficacy.

10.1. Plant extracts for kefir enrichment

Incorporating medicinal plant extracts and essential oils into kefir represents an effective approach for fortifying the beverage with bioactive compounds, capitalizing on their Generally Recognized as Safe (GRAS) status. This fortification has been shown to enhance kefir's functional properties, though it may sometimes impact sensory acceptance. Below are notable studies exploring various plant-based additives for kefir enrichment:

- **Ginger and cinnamon extracts:** In a study by Setiyoningrum et al. (2019), goat milk kefir enriched with ginger and cinnamon extracts showed no significant changes in protein content, pH, total titrated acid, or microbial content. However, kefir with 8% (v/v) cinnamon extract displayed a substantial increase in antioxidant activity (+12.4%). Despite this improvement in antioxidant levels, the use of both extracts negatively impacted sensory outcomes (Setiyoningrum, Priadi, and Afiati 2019).

- **Flaxseed extract:** Kim et al. demonstrated the positive impact of flaxseed (*Linum usitatissimum*) extract on lactic acid bacteria growth in kefir. Flaxseed extract, known for its α -linolenic acid, lignans, and fiber content, stimulated the growth of strains like *Lactobacillus kefiranofaciens* DN1, *Lactobacillus bulgaricus* KCTC3635, *Lactobacillus brevis* KCTC3102, and *Lactobacillus plantarum* KCTC3105. Treating these bacteria with 100 μ L/mL of crude flaxseed extract significantly boosted their growth compared to the control, indicating that flaxseed could serve as a prebiotic to support probiotic development in kefir (Kim et al. 2017).
- **Hazelnut milk:** Atalar's research (Atalar, 2019) on kefir fortified with hazelnut milk at 25%, 50%, and 75% showed enhanced bioactive properties, including higher levels of phenolic compounds and antioxidant capacity. The addition of hazelnut milk, especially at 50-75%, improved the organic acid profile and viability of lactobacilli and lactococci. Notably, this enrichment led to a decrease in lactic and citric acid levels while significantly increasing malic and acetic acids, thereby altering the flavor profile and increasing nutritional value
- **Honey and rosemary essential oil:** Perna et al. studied donkey milk kefir enriched with honey (30% w/v) and rosemary essential oil (0.15% w/v). Kefir with essential oil showed heightened antioxidant activity, while honey-fortified kefir exhibited stronger ferric-reducing antioxidant power (FRAP). Antioxidant activity increased during refrigerated storage, peaking at 15 days. Sensory analysis revealed consumer preference for honey-enriched kefir over rosemary oil-enhanced versions (Annamaria, Simonetti, and Gambacorta 2018).
- **Green and black tea:** Enrichment of kefir with green and black tea at 2.0% or 4.0% (w/v) enhanced the product's antioxidant potential. Green tea-fortified kefir displayed superior antioxidant properties over black tea-enriched kefir, with higher concentrations (4%) yielding more pronounced benefits. This fortification could produce a functional dairy beverage with improved health benefits, and a 2.0% green tea concentration—a level equivalent to a typical cup of tea—also contributes positively to kefir's taste (Karagozlu et al. 2017).

10.2. Kefir fortification with juices and honey

Fortifying kefir with fruit juices and honey represents a practical approach to enhancing its antioxidant capacity, extending shelf life, and improving palatability. A recent study explored the effects of adding various fruit juices—black carrot, black mulberry, pomegranate, and strawberry—to kefir in concentrations of 10%, 25%, and 50% (w/w), followed by storage at 4°C for 12 weeks to assess antioxidant activity, sensory attributes, and stability. Key findings from this research include:

- **Enhanced antioxidant properties:** The addition of black mulberry, pomegranate, and strawberry juices significantly improved the antioxidant levels in kefir. These fruits are rich in

polyphenols, vitamins, and anthocyanins, which contribute to a heightened antioxidant profile in the kefir, supporting health benefits such as reduced oxidative stress.

- **Improved sensory characteristics:** Kefir samples enriched with pomegranate and strawberry juice showed notable improvements in sensory qualities, such as taste and aroma, which could increase consumer acceptance. The fruity flavors may complement kefir's natural tang, enhancing its appeal.

- **Stability of anthocyanins:** Black mulberry-enriched kefir exhibited the highest stability of anthocyanins during storage. Anthocyanins, responsible for vibrant colors and antioxidant effects, are often sensitive to degradation; their stability in black mulberry-enriched kefir suggests potential for creating visually appealing, health-benefiting products with extended color and nutrient retention.

Table 3: The fortification effect of kefir with different extracts (Aiello et al., 2020)

Extract	Enrichment	Effect
Kefir + vegetable juices	4% (w/v)	↑ Alcohols ↑ Antioxidant activities ↑ Volatile compounds
Papaya juice + LAB	45% (w/v)	↑ Antioxidant activities ↑ Aroma associated compounds
Kefir + pomegranate juice	15% (w/w)	↓ pH values ↑ Acidity ↓ Colorimetric parameters
Kefir + honey	3% (w/w)	↓ Acidity ↓ Colorimetric parameters ↑ Viscosity ↑ Sweetness

↑: increase ↓: decrease

Enriching kefir with fruit juices provides a dual benefit of improved taste and enhanced nutritional value, particularly in antioxidant activity. This approach leverages the natural qualities of fruits to support functional dairy innovations, appealing to health-conscious consumers (Kabakcı, Türkyılmaz, and Özkan., 2020).

10.3. Kefir fortification with Agro-Food waste extracts

The use of agricultural and agro-industrial waste extracts for kefir fortification presents a sustainable approach to food innovation, addressing both environmental and economic concerns. Agro-food waste, which includes by-products from fruit, vegetable, and grain processing, is rich in valuable bioactive compounds such as polyphenols, antioxidants, fibers, and vitamins. These metabolites not only contribute to reducing waste but also offer potential health benefits when used in food products. Key benefits include:

- **Enhanced nutritional profile:** Secondary metabolites from agro-food waste, like antioxidants and polyphenols, can increase kefir's health benefits. These bioactive compounds

have been associated with reducing oxidative stress, supporting immune function, and contributing to anti-inflammatory effects.

- **Sustainability and waste reduction:** Utilizing food processing by-products as kefir fortifiers provides a sustainable solution by reducing the volume of waste that might otherwise contribute to environmental pollution. It also promotes a circular economy approach, where waste materials are repurposed to create value-added products.

- **Cost-effectiveness:** Agro-food waste is often readily available and inexpensive, making it a cost-effective ingredient for enhancing functional foods. This approach not only reduces waste management costs but also creates an economic incentive for producers to develop functional foods that are both affordable and nutritionally beneficial.

- **Functional food innovation:** Incorporating these extracts into kefir may introduce new flavors, and health-promoting properties, aligning with the consumer demand for functional foods. For instance, phenolic-rich extracts from fruit peels or seeds can provide an enhanced antioxidant profile, while fiber-rich components may contribute to improved gut health.

Table 4: The Effect of kefir fortification with Agro-Food waste extracts (Aiello et al., 2020)

<i>Extract</i>	<i>Enrichment</i>	<i>Effect</i>
<i>Kefir + wine pomace</i>	0.1% (w/v)	↑ Antioxidant activities ↑ Inhibitory activity: α -amylase, α -glucosidase, pancreatic lipase
<i>Kefir + pine bud syrup</i>	2–10% (w/v)	↑ Sensorial parameters

In developing value-added kefir products, researchers have explored incorporating ingredients such as pine bud syrup and dietary fibers with stabilizers.

- **Pine bud syrup fortification:**

- *Proposal and effects:* One proposal involves adding pine bud syrup, rich in polyphenols and terpenes, at concentrations between 2% and 10% (w/w). This syrup is known for its high antioxidant activity. Including it in kefir resulted in an increase in total solids and a decrease in fat content, protein levels, and pH.

- *Sensory outcomes:* Among the samples, kefir with 10% pine bud syrup achieved the highest sensory ratings, reflecting positive consumer acceptance. This fortification aligns with trends in functional foods, where high antioxidant content enhances both health benefits and consumer appeal (Aiello et al., 2020).

- **Fortified sweetened milk kefir with dietary fibers and stabilizer:**

- *Research setup:* Another study aimed at prolonging kefir's shelf life and maintaining texture involved fortifying sweetened milk kefir with stabilizers and dietary fibers (soya fiber, oat fiber, and inulin). The fortified kefir with stabilizer (WS) was created by adding 0.1% pectin, 6%

sugar, and 3% inulin. The milk underwent a heat treatment at 90-92°C for 10 minutes, then cooled to 30°C. Kefir grains were added at 4 g/L, and the mixture was incubated at 30°C for 20-24 hours to reach a titratable acidity of 1% lactic acid.

-Comparative analysis: For comparison, a control kefir was prepared without pectin, sugar, and inulin. Additionally, a version without stabilizer (WOS) included only sugar and inulin.

-Physicochemical, sensorial, and textural benefits: The stabilizer (pectin) helped enhance the texture and sensory qualities of the kefir over storage, ensuring a smoother consistency and maintaining sweetness, potentially increasing shelf life. By reducing syneresis and maintaining stability, pectin and dietary fibers support a desirable mouthfeel, indicating that stabilizers may be useful in producing consistent, high-quality kefir with prolonged storage appeal (Solanki, Ghosh, and Kumawat., 2023).

Shelf life enhancement and fortification innovations in kefir

Recent studies emphasize the benefits of fortifying kefir with natural ingredients and stabilizers to extend its shelf life and improve sensory qualities. Key findings from recent research underscore the potential of plant-based additives, stabilizers, and innovative preservation techniques in enhancing kefir's physicochemical, textural, and storage properties:

- **Fortified sweetened kefir with stabilizer (WS):**

- Superior storage and quality: Fortified sweetened kefir (WS), enhanced with pectin and dietary fibers, displayed significantly better physicochemical, sensory, and textural properties compared to control kefir. While control kefir was deemed unfit for consumption by the 12th day due to the overactive yeast and tangy flavor, fortified sweetened kefir (WS) remained safe and enjoyable up to the 15th day.

- Textural benefits: The use of pectin as a stabilizer helped maintain smooth texture and minimize syneresis (whey separation) during storage, making fortified kefir more consistent and palatable over an extended period. These results suggest that stabilizers like pectin, combined with fibers, can improve shelf life, quality, and consumer appeal in fermented dairy products (Solanki, Ghosh, and Kumawat., 2023).

- **Plant-based natural preservatives for kefir preservation:**

- Plant components for antioxidant and antibacterial activity: A prominent research direction in the dairy industry focuses on utilizing plant-based additives with natural antioxidant and antibacterial properties to serve as preservative alternatives to traditional methods. This approach aims to reduce oxidation and improve kefir's shelf stability, ensuring that the product remains safe and flavorful over time.

- *Innovative organic preservative mix*: A specific method involves incorporating a 0.5% (w/w) mixture of powdered *Cladonia* lichens, lingonberries, and bearberry leaves into ready-to-use kefir. This powder is produced by mechanically activating the plant mixture at high speeds in a ball mill, creating a potent, solvent-free preservative. Studies demonstrate that this addition can extend kefir's shelf life up to 16 days without altering its taste, providing an eco-friendly and cost-effective solution. This technique is advantageous for its simplicity, environmental sustainability, and preservation efficacy, ensuring the natural flavor and safety of the product over time (Stepanovova and Chirikova., 2021). These advancements highlight the promising potential of natural plant-based and stabilizer-enhanced methods for fortifying kefir.

10.4. Kefir enriched with encapsulated volatile oils

A recent study examined the effects of enriching cow's milk kefir with encapsulated volatile oils—specifically fennel, mint, and lavender—using enzymatic methods. The primary aim was to analyze the chemical composition and antimicrobial activity of these oils when encapsulated in sodium alginate, which helps stabilize the volatile oils against environmental degradation from factors like heat, light, and humidity.

10.4.1. Protection and gradual release of bioactive compounds

- *Encapsulation benefits*: Sodium alginate encapsulation protects the volatile oils, ensuring their bioactive compounds are gradually released in kefir. This controlled release maintains the oils' functional benefits over time, even during storage, while preventing the rapid evaporation or degradation often associated with volatile oils.

- *Improved stability*: Encapsulated oils are shielded from oxidation, extending the shelf life and preserving the efficacy of bioactive compounds. This approach is especially beneficial for oils like lavender and mint, which are sensitive to environmental conditions but offer robust antimicrobial and antioxidant properties.

10.4.2. Antimicrobial activity enhancement

The study found that encapsulated volatile oils enhanced the antimicrobial properties of kefir, adding a protective layer against certain pathogens. Fennel, mint, and lavender oils demonstrated antimicrobial effectiveness, likely contributing to improved shelf life and product safety. This activity helps inhibit spoilage bacteria and extends the product's quality period.

10.4.3. Enhanced chemical composition

- *Chemical profile impact*: The presence of encapsulated oils altered the chemical composition of kefir by introducing essential oils' bioactive constituents, which are known for their therapeutic effects, such as digestive support (fennel), calming properties (lavender), and cooling effects (mint).

- *Sensory and functional benefits:* Besides the antimicrobial advantages, the oils may also enhance the sensory profile of kefir with subtle flavor notes, although encapsulation helps moderate the release to avoid overpowering the natural taste of kefir.

Enriching kefir with encapsulated volatile oils illustrates a sophisticated fortification technique that combines enhanced functionality, antimicrobial benefits, and the gradual release of beneficial compounds. Additionally, the antimicrobial properties of volatile oils help prolong the shelf life of these products, ensuring their quality and safety over an extended period (Tița et al., 2023). Four variations of kefir were prepared for analysis: kefir enriched with encapsulated lavender volatile oil, kefir enriched with encapsulated mint volatile oil, kefir enriched with encapsulated fennel volatile oil, and a control sample without volatile oils. The study involved evaluating these samples at three different storage intervals: the first day, the 10th day, and the 20th day.

The three kefir samples enriched with encapsulated lavender, mint, and fennel volatile oils demonstrated superior performance compared to the control sample throughout the analysis period. This suggests that the use of medicinal and aromatic plants in food preparation offers a promising alternative due to their antioxidant properties, which can contribute to consumers' overall health benefits and potentially extend the product's shelf life by incorporating bioactive compounds (Tița et al., 2023). The addition of orange juice enhanced the fermentation process, improving kefir grains' ability to ferment pomegranate juice and increasing the survival rates of lactic acid bacteria (LAB) during storage at 4°C over 4 weeks. Notably, 75% of LAB cells survived (6.48 log CFU/mL) in the mixed substrate after 4 weeks, in contrast to 24% survival in plain pomegranate juice. Lactic acid formation, an indicator of metabolic activity, was observed in all fermented products, with the highest levels recorded in the mixed substrate (1.3–1.9 g/L). Sugar consumption and ethanol production were tracked during fermentation, indicating controlled fermentation suited for creating low-alcohol beverages (Kazakos et al., 2016).

III. Materials and Methods

Kefir, a fermented dairy product, is notably susceptible to changes during storage, with its physicochemical properties and microbial composition evolving over time. These changes can impact kefir's texture, sensory attributes, and overall quality, underscoring the need for comprehensive monitoring to ensure safety and quality. This chapter outlines the methods and techniques applied in this study, organized into seven primary areas:

1. **Materials and methods for kefir production:** Details the ingredients, kefir grain preparation, and production protocol used to ensure consistency across all samples.
2. **Shelf-life study:** Describes the storage conditions, including temperature and duration, alongside parameters tracked to assess shelf-life, such as microbial stability, physicochemical properties, and sensory characteristics.
3. **Physicochemical analyses:** Comprises measurements of pH, titratable acidity, syneresis (whey separation), and proteolysis. These analyses provide insights into kefir's chemical stability, acidity changes, protein breakdown, and water-holding capacity over time.
4. **Microbiological analyses:** Involves enumeration of lactic acid bacteria, yeasts, molds, and mesophilic bacteria to monitor microbial load and diversity throughout storage, ensuring that kefir maintains its probiotic and safe-to-consume status.
5. **Rheological and textural analyses:** Measures viscosity, firmness, cohesiveness, and consistency to track changes in texture and mouthfeel. These properties are key indicators of consumer acceptability and product stability.
6. **Sensory evaluation:** Describes the sensory panel and analysis protocol, including the criteria used to evaluate taste, aroma, texture, and appearance. Sensory feedback provides a direct assessment of consumer perception and product quality.
7. **Statistical methods:** Outlines the statistical techniques used to analyze data, ensuring that observed changes are significant and meaningful. This section includes approaches for evaluating result significance, such as ANOVA, t-tests, and regression analyses.

This comprehensive approach offers a detailed view of kefir's quality changes during storage, providing essential information for optimizing kefir production, storage, and commercialization.

1. Kefir production

The production of kefir follows five essential steps:

- **Grain activation:** Kefir grains, which contain a symbiotic community of lactic acid bacteria, yeasts, and other microorganisms, are first activated. This involves soaking the grains in

fresh milk for a specified period (typically 24-48 hours) to initiate microbial activity. Activation is essential for ensuring that the grains are ready to ferment.

- **Kefir preparation:** After activation, the kefir grains are combined with fresh milk in a specific ratio, commonly 5-10% kefir grains to milk by weight. This mixture provides the environment for fermentation, where the grains metabolize the milk's lactose, producing lactic acid, carbon dioxide, and minor amounts of alcohol.
- **Incubation:** The mixture is incubated at a controlled temperature, typically between 20-25°C, for 12-24 hours. During this phase, fermentation occurs, transforming the milk's texture, flavor, and acidity. The incubation period is closely monitored to achieve the desired thickness, taste, and microbial content.
- **Filtration:** After incubation, the kefir grains are separated from the fermented milk by filtration. The grains are either reused for the next batch or stored for future use. Filtration ensures that the final product has a smooth consistency without grains.
- **Storage:** The filtered kefir is then transferred to sterile containers and stored under refrigerated conditions, generally at 4°C. Cold storage slows down microbial activity, preserving the kefir's quality, taste, and texture over its shelf life.

1.1. Grain activation

Kefir grains were sourced from local kefir users and stored in the laboratory for later use. Before each use, the grains were sieved and rinsed with distilled water, followed by inoculation into goat's milk for activation. The activation process involves several steps to ensure the grains are fully reactivated and prepared for consistent kefir production:

- **Defrosting and preparation** First, the kefir grains are taken out of the freezer and allowed to defrost at room temperature. Once thawed, they are carefully rinsed with distilled water to remove any residual substances from previous storage.
- **Initial inoculation:** After defrosting and washing, the grains are slightly dehydrated and then added to cold pasteurized milk, which serves as the initial fermentation substrate.
- **Incubation for activation:** The milk-grain mixture is placed in an incubator set to 20-25°C and left for 24 hours. During this period, the grains begin metabolizing the milk, initiating microbial activity essential for effective fermentation.
- **Straining and refreshing:** After each 24-hour period, the mixture is strained to remove the grains, and the used milk is discarded. Fresh milk is added, and the process is repeated, typically daily, for 3 to 7 days.

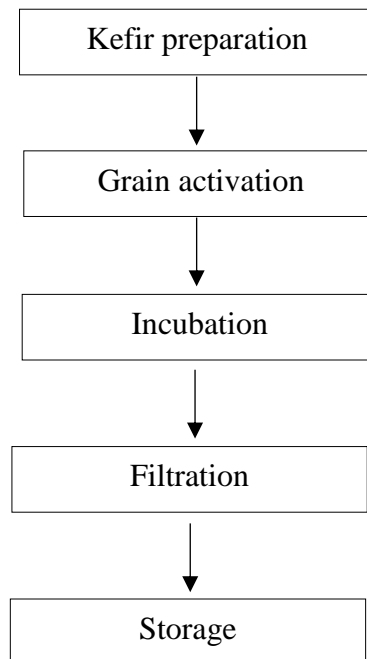


Figure 2: Goat's milk kefir production diagram

- **Gradual increase in milk volume:** If the milk begins to thicken within 24 hours, indicating active fermentation, the milk volume is gradually increased in subsequent cycles. This adjustment allows the grains to adapt and fully reactivate, ensuring they can ferment larger milk volumes effectively. This daily activation cycle prepares the kefir grains, restoring their microbial vigor and optimizing them for regular kefir production.

1.2. Kefir preparation

Kefir production follows the activation of kefir grains and requires precise control of milk volume, kefir grain percentage, temperature, and incubation time. In the first part of this study, we experimented with various combinations of these variables - specifically, the kefir grain/milk ratio (%), incubation temperature (°C), and incubation time (hours). After determining the optimal combination (or “best triplet”), these standardized conditions were used in the second phase of the study. Following the identification of this ideal triplet of variables, we prepared two types of kefir: a control sample without lemon extract and a flavored sample with added lemon extract. This dual approach allowed for a comparative analysis between the standard kefir and the lemon-enhanced version, enabling us to evaluate the impact of lemon extract on the physicochemical, sensory, and microbial characteristics of kefir (Table 5).

Table 5: The different parameters of the two types of kefir

Kefir	Ratio (%)	Extract dose (%)	Incubation time (h)	Incubation temperature (°C)
Control	0.9	0	24	20
Flavored	0.9	1	24	20

1.3. Incubation and filtration

Incubation involves placing the prepared kefir mixture in an incubator set to a specific temperature for a designated period. This step is crucial for optimizing the fermentation process by controlling the environmental conditions that influence kefir's microbial activity, texture, and flavor. For the first phase of this study (the "Fixation Triplet"), we applied a Box-Behnken experimental design (Table 7) to determine the optimal combination of variables. Three factors were chosen for investigation:

- **R** (Ratio of kefir grains to milk),
- **T** (Temperature in °C), and
- **t** (Incubation time in hours).

Each factor was tested at three levels: low (-1), central (0), and high (+1), resulting in a 14-treatment experimental matrix. This design allowed us to systematically evaluate the influence of each variable and their interactions, ultimately guiding the selection of the ideal conditions for kefir incubation (Table 6).

Table 6: Factors and their different values

<i>Factors</i>	<i>Low (-1)</i>	<i>Central (0)</i>	<i>High (+1)</i>
<i>Ratio (%)</i>	0.5	1	1.5
<i>Temperature (°C)</i>	15	20	25
<i>Time (h)</i>	16	20	24

Table 7: Experimental Design

#	run. order	std. order	Ratio	Time	Temperature
1	1	5	0.5	20	15
2	2	11	1.0	16	25
3	3	8	1.5	20	25
4	4	1	0.5	16	20
5	5	6	1.5	20	15
6	6	10	1.0	24	15
7	7	2	1.5	16	20
8	8	13	1.0	20	20
9	9	4	1.5	24	20
10	10	7	0.5	20	25
11	11	14	1.0	20	20
12	12	12	1.0	24	25
13	13	9	1.0	16	15
14	14	3	0.5	24	20

Factors levels– kefir: 0.5, 1.0 and 1.5%– Time: 16, 20 and 24 hours– Temp: 15, 20, 25 °C

For the second part of the study (the **Monitoring of Shelf-Life**), we focused on a single treatment identified as optimal based on the results from the initial analysis. This selected treatment, having shown the most favorable combination of variables (R, T, and t) during the incubation phase, was used consistently to evaluate the kefir's quality over its storage period. Monitoring this specific treatment allowed for a detailed assessment of the shelf-life, providing insights into changes in physicochemical, sensory, and microbial properties under optimal production conditions. Filtration, as the name suggests, is the process of filtering the kefir at the end of its fermentation (incubation) to separate the kefir grains from the liquid. This step yields the final kefir product, free from grains, and ensures a smooth consistency suitable for consumption.

1.4.Storage

Storage conditions in this research varied across the two parts of the study:

- **First part (Experimental treatments):** All 14 treatments were stored at 4°C during the physicochemical, textural, and microbiological analyses. This consistent storage temperature allowed for a controlled comparison of the 14 different treatment conditions.
- **Second part (Shelf-life monitoring):** For the shelf-life study, the single treatment identified as optimal (based on the best combination of variables) was stored at 4°C for a duration of 13 days. This storage period enabled us to monitor changes in quality parameters over time under optimal conditions, providing insights into the product's shelf-life and stability.

2. Monitoring of shelf life of control and flavored kefir

Two types of kefir were produced for shelf-life monitoring: a control kefir, traditionally made by inoculating milk with kefir grains, and a flavored kefir, created by adding lemon extract to the milk and grains before fermentation. The shelf life of both kefir types was monitored over a 13-day period, assessing various physicochemical, textural, and microbiological parameters. This systematic approach provided a detailed evaluation of the stability and quality changes in each kefir type over time, offering valuable insights into their shelf life characteristics under controlled storage conditions.

Table 8: Analysis planning

Analysis /Day	0	1	2	3	4	5	6	7	8	9	10	11	12	13
Physicochemical + Textural														
Microbiological														

2.1.Physicochemical analysis

To ensure the physicochemical quality of each kefir treatment, several tests were conducted.

These included:

- **pH measurement:** To monitor acidity levels and fermentation progress.
- **Acidity analysis:** To assess the overall sourness and lactic acid concentration.
- **Syneresis test:** To evaluate the water-holding capacity, indicating product stability and texture.
- **Proteolysis evaluation:** To measure protein breakdown, which can impact flavor, texture, and nutritional value.

These analyses provided a comprehensive profile of the kefir's physicochemical properties, essential for assessing product quality and stability.

2.1.1. Hydrogen potential (pH)

The pH of kefir was measured using a pH meter (model) to monitor its acidity level. The procedure began with calibrating the pH meter using two standard buffer solutions at pH 4.00 and pH 7.00 to ensure accuracy. A sample of kefir was then poured into a clean container, and the pH meter electrode (model) was immersed in the liquid. The reading was allowed to stabilize, after which a second measurement was taken to confirm accuracy. The final pH value was recorded, providing an indicator of the kefir's acidity and fermentation stage.

2.1.2. Titratable acidity (TA)

Titratable acidity (TA) of kefir was measured by titration with sodium hydroxide (NaOH) in the presence of phenolphthalein as an indicator, which causes the solution to change from white to pink at the endpoint. The procedure was as follows:

- **Sample preparation:** A 10 mL sample of kefir was measured and placed into a clean beaker.
- **Indicator addition:** Two drops of phenolphthalein were added to the sample.
- **Titration:** The sample was titrated with 1/9 N NaOH (Dornic solution), where 1 mL of this NaOH solution corresponds to 0.01 grams of lactic acid (AFNOR, 1980). The endpoint was reached when a stable pink color appeared. The titratable acidity (°D) was calculated using the formula:

$$TA (^{\circ}D) = V_i * 10$$

where V_i represents the volume of NaOH (in mL) required to neutralize the sample.

The Dornic degree (°D) indicates the amount of lactic acid per liter of sample, with one °D representing one decigram of lactic acid per liter of kefir. This measurement provides an indicator of the kefir's lactic acid content and overall acidity, essential for assessing fermentation progress and product quality. To ensure accuracy, this titratable acidity measurement can be repeated three independent times. The average of these three values is then

calculated and recorded as the final result, providing a reliable indicator of the kefir's lactic acid content and acidity level (Allioui, Bouchehed, and LAKHAL 2021).

2.1.3. Syneresis

Syneresis in kefir was measured following the method of Schmidt and Bouma (1992). The procedure involves separating and quantifying the amount of free whey, which reflects the extent of whey separation—a key quality parameter in fermented dairy products. Here's the method:

- **Sample preparation:** A 20 g sample of kefir was prepared.
- **Centrifugation:** The sample was degreased by centrifuging it at 3000 rpm for 20 minutes at 4°C. This step separates the solid and liquid phases.
- **Filtration:** The centrifuged sample was then filtered to isolate the liquid whey.

Syneresis was expressed as a percentage of free whey, calculated using the following formula:

$$\text{Free whey}(\%) = \frac{\text{PFSW} * 100}{\text{ISW}}$$

where PFSW represents the Post filtration Sample Weight and ISW the Initial Sample Weight (in gram). This method provides a standardized measure of syneresis, allowing for consistent comparisons between different kefir samples or production batches. By quantifying whey separation, it offers valuable insights into kefir's stability, texture, and overall quality.

2.1.4. Proteolytic activity

To measure proteolytic activity in kefir, the following steps are used to isolate the proteins for analysis:

- **pH adjustment:** Adjust the kefir sample's pH to 4.6, the isoelectric point of casein, by adding a 1 M HCl solution. This step helps precipitate the casein, allowing separation of the whey, where proteolytic enzymes are active.
- **Centrifugation:** Centrifuge a 10 mL sample at 3000 rpm for 20 minutes at 4°C. This process separates the liquid (whey) from the solid components.
- **Whey separation:** After centrifugation, carefully extract the whey using a micropipette.
- **Filtration:** Filter the separated whey to remove any residual solids, ensuring that only the liquid phase is analyzed for proteolytic activity.

This procedure isolates the whey, which can then be tested to determine the extent of protein breakdown in kefir, an important factor in assessing the texture, flavor, and nutritional quality of the product. To quantify proteolytic activity in kefir, the following steps are conducted:

1. Initial absorbance measurement:

- Dilute 1 mL of whey in 100 mL of distilled water.

- Measure the absorbance at 280 nm using distilled water as a blank standard.

2. Heat treatment:

- Boil the remaining whey sample in a water bath for 10 minutes (refer to Fig. 18 for setup).
- After boiling, centrifuge the sample at 3000 rpm for 20 minutes at 4°C and filter the resulting liquid.

3. Post-boiling absorbance measurement:

- Dilute 1 mL of the filtered, boiled whey in 10 mL of distilled water.
- Measure the absorbance at 280 nm, again using distilled water as a blank.

4. Proteolytic activity calculation:

$$\text{Proteolytic activity} = \text{Filtrate absorbance before boiling} - \text{Filtrate absorbance after boiling}$$

This calculation represents the proteolytic activity in the kefir sample, as it measures the release of tyrosine and tryptophan, which absorb at 280 nm, from proteins due to enzymatic activity. This method provides an estimate of protein breakdown in kefir, indicating the extent of proteolysis and its impact on texture and flavor.

2.2. Textural analysis

Texture is a critical attribute of kefir, influencing its technological quality, consumer appeal, and shelf stability. Therefore, monitoring textural parameters such as firmness, viscosity index, cohesiveness, and consistency is essential.

The texture of kefir was evaluated using a compression test on a Texturometer (TA.XT Plus Texture Analyzer) equipped with a 5 kg load cell. The kefir sample was placed in a jar, positioned horizontally, and centered under the probe before beginning the measurement. The following conditions were used:

- **Probe and speed:** A flat cylindrical probe (P/36R, diameter 36 mm) was used, operating at a speed of 2 mm/s.
- **Temperature:** All measurements were conducted at room temperature to ensure consistency.
- **Data acquisition:** Texture Exponent TPA32 software was used alongside the Texture Profile Analysis (TPA) test, which involved two compression cycles to mimic the chewing process.

This method allowed for the quantification of multiple textural parameters:

- **Firmness:** Resistance to deformation, reflecting kefir's structural integrity.
- **Viscosity index:** The flow resistance of the kefir, indicating its thickness.
- **Cohesiveness:** The extent to which kefir retains its structure upon compression.
- **Consistency:** The uniformity and stability of texture throughout the sample.

These textural measurements provide insight into the quality, stability, and mouthfeel of kefir, essential for assessing its consumer acceptability and storage performance.

2.2.1. Firmness

Firmness refers to the resistance of the kefir sample to deformation when a force is applied. It indicates the structural integrity of the product, which is essential for consumer experience.

2.2.2. Index of viscosity

The viscosity index measures kefir's resistance to deformation at a specific rate, reflecting the product's thickness and flow properties.

2.2.3. Cohesiveness

Cohesiveness is the ratio of the area under the curve for the second compression to that of the first compression (Surface 2/Surface 1). This parameter reflects the kefir's ability to maintain its structure after initial deformation, indicating its resilience and chewiness.

2.2.4. Consistency

Consistency in kefir refers to the overall texture and flow properties of the product, which are influenced by several factors including firmness, viscosity, and cohesiveness.

2.3. Microbiological analysis

To ensure the microbiological quality of kefir, monitoring of various bacteria and yeast populations was performed to confirm that the product remains stable and safe for consumption. Conventional microbiological techniques were employed, and all samples were prepared following the French standard NF V08-010 (1996).

2.3.1. Preparation of material for sterilization

To prepare for the analysis of lactic acid bacteria and mesophiles at each dilution level, all necessary materials were gathered and sterilized. This included Petri dishes, boxes of micropipette tips, tubes, and glass bottles for preparing culture media. Sterilization was carried out in an autoclave at 121°C for 15 minutes to ensure aseptic conditions.

This setup ensures that all tools and containers are sterile and organized, facilitating efficient preparation and inoculation of culture plates. This meticulous preparation allows for accurate dilution series and reliable microbial analysis, ensuring the kefir's microbiological safety.

2.3.2. Preparation of culture medium

The preparation of the culture medium involved the following steps:

- **Ingredient measurement:** The required quantities of each ingredient were precisely weighed.
- **Addition of distilled water:** Distilled water was added to the weighed ingredients to reach the desired volume.
- **Dissolution:** The mixture was agitated until all components were completely dissolved.

- **Sterilization process:** The prepared solution was sterilized in an autoclave at 121°C for 15 minutes to eliminate potential contaminants and ensure a sterile environment.

- **Temperature maintenance:** Post-sterilization, the culture medium was held at 50°C until use, maintaining its liquid state for ease of handling and preventing contamination. This temperature also preserved the medium's nutritional integrity, supporting optimal conditions for microbial growth.

For lactic acid bacteria: For the cultivation and enumeration of lactic acid bacteria, MRS agar (de Man, Rogosa, and Sharpe agar) was utilized. MRS agar is specifically formulated to support the growth of *Lactobacillus* species and other lactic acid bacteria, which often have stringent nutrient requirements. This medium contains essential nutrients and growth factors tailored to promote the development of lactic acid bacteria, ensuring optimal conditions for their isolation and quantification.

For mesophilic bacteria: For the enumeration of mesophilic bacteria, PCA (Plate Count Agar), also referred to as PCMA (Plate Count Milk Agar), was employed. This medium is commonly used to quantify total aerobic mesophilic bacteria in various sample types, including food and dairy products. PCA provides a non-selective environment suitable for the growth of a broad spectrum of mesophilic organisms, facilitating an accurate assessment of microbial load.

2.3.3. Preparation of dilutions

For microbiological enumeration, serial dilutions were prepared as follows:

- **Materials preparation:** Tubes containing 9 mL of sterile diluent (Buffered Peptone Water, BPW) and sterile pipettes were organized prior to the procedure.

- **Initial dilution:** A 1 mL aliquot of the initial sample was transferred into a tube containing 9 mL of BPW to achieve a 10^{-1} dilution. The tube was mixed thoroughly to ensure even distribution of microorganisms.

- **Subsequent dilutions:** Using a fresh sterile pipette for each dilution step, 1 mL from each prior dilution was transferred to a new 9 mL tube, mixed thoroughly to reach the next dilution level. This process was repeated until the desired dilution was achieved.

- **Homogeneity assurance:** Each tube was shaken well after each transfer to maintain a uniform distribution of microorganisms within the diluent, minimizing variability in microbial counts. This methodical dilution procedure ensured accuracy in the microbiological analysis and prevented cross-contamination, enabling reliable enumeration of bacterial and yeast populations.

2.3.4. Preparation of Petri dishes for LAB and MES and incubation

To cultivate and enumerate mesophilic bacteria (MES) and lactic acid bacteria (LAB), the following procedures were conducted:

- **Mesophilic bacteria (MES):**

- **Inoculation:** One milliliter of each dilution was added to a sterile Petri dish, followed by the addition of Plate Count Agar (PCA; Sharlau) as the culture medium. The medium was poured over the sample to achieve a depth culture.

- **Incubation:** The inoculated, inverted Petri dishes were incubated at 37°C for 48 hours, following the standard protocol (NF V08-011, 1998). This incubation period and temperature ensured optimal conditions for the growth of mesophilic bacteria.

- **Lactic acid bacteria (LAB):**

- **Culture Medium:** Man Rogosa and Sharpe agar (MRS; Frilabo 610024) was used, with 1 mL of Tween 80 (80031) added per liter of medium to enhance LAB growth.

- **Double Layer Technique:** Inoculation was performed in a double layer, where a top layer of bacteriological peptone agar (Liofilchem) was added to create anaerobic conditions conducive to LAB growth.

- **Incubation:** The prepared dishes were incubated at 30°C for 48 hours, providing the appropriate environment for LAB proliferation. These tailored procedures ensured optimal growth conditions for each bacterial type, allowing for accurate enumeration and a reliable assessment of the microbiological quality of the kefir samples.

2.3.5. Yeasts and molds count

Yeast and mold counts were performed using 3M Petrifilm™ Yeast and Mold Count Plates, which contain a colored indicator that differentiates between yeast and mold colonies, enhancing visibility and ease of reading. Incubation times ranged from 3 to 5 days at 25°C, with sample dilutions from 10⁰ to 10⁻². The procedure for yeast and mold counting was as follows:

- **Sample application:** The Petrifilm was placed on a flat surface, and the top film was lifted. placing, 1 mL of the diluted sample on the center of the bottom film.
- **Spreading the sample:** The top film was released without rolling, and the 3M™ Petrifilm™ Yeast and Mold Spreader was positioned in the center of the plate. Light pressure was applied to evenly spread the sample within the circular area, ensuring coverage without twisting or sliding the spreader.
- **Incubation:** The prepared Petrifilm plates were incubated face up at 25°C ± 1°C for 3 to 5 days, depending on the sample and desired colony maturity.

- **Counting colonies:** Following incubation, yeast and mold colonies were counted using a standard colony counter or an illuminated magnifier. Yeast colonies appeared as small, defined colonies with a blue-green color, while mold colonies were typically larger, more diffuse, and displayed variable colors.
- **Bacterial count:** Bacterial colony enumeration was conducted using an electronic colony counter.

2.4. Sensory analysis

2.4.1. Preparation of the response sheet

To quantify subjective sensory experiences in a way that allows for comparisons between samples, consumer preference assessments, and product quality evaluations, a response sheet (see Annex) was prepared for sensory evaluation of two coded kefir samples (Control and Flavored). A 9-point non-structural scale was employed, ranging from 1 ("Dislike extremely") to 9 ("Like extremely"), to evaluate specific quality attributes. The evaluation process was divided into three steps:

- **Appearance:** Participants visually observed the samples.
- **Odor:** Participants assessed the aroma of each sample.
- **Taste:** Participants rated overall taste, sourness, smoothness, and overall acceptance.

The response sheet included two rows for each sensory attribute, one for each kefir sample, where participants marked their ratings on the non-structural scale. Spaces were provided for sample codes to maintain anonymity and eliminate bias.

2.4.2. Preparation of samples

For the sensory evaluation, two 1-liter samples of kefir were prepared using the optimized conditions identified in shelf-life monitoring:

- **Control kefir:** Produced with 0.9% kefir grains/milk ratio, fermented at 20°C for 24 hours.
- **Flavored kefir:** Also produced with a 0.9% grains/milk ratio, with an additional 1% lemon extract flavor, fermented under the same conditions.

The sensory analysis was conducted with 45 participants. Each participant was provided with coded samples of both control and flavored kefir. Following tasting, each participant completed a response sheet by marking their ratings on the non-structural scale. Upon collection, the distances from the left edge of the scale to each mark were measured in cm using a ruler, allowing for precise, quantitative data collection. The collected data were subsequently organized and analyzed statistically to assess differences between the control and flavored kefir samples, identifying any significant effects of flavor addition on sensory attributes.

3. Statistical analysis

All the data obtained in this study were analyzed using the R software (R Studio 2023.03.1). A breakdown of the methodology follows.

- **Data organization and import:** Experimental data were initially organized in an Excel file and then imported into RStudio for analysis.
- **First Part: Response surface model**

A response surface model was fitted to explore the relationships between factors and responses, allowing for optimization of experimental conditions. This was done using the package *rsm* and solving for an analysis of variance (ANOVA) with a $\alpha=0.05$. - The model was analysed by examining coefficients, p-values, and adjusted R^2 values. Response surfaces were visualized using contour plots or 3D surfaces to optimize the physicochemical, textural, and microbiological qualities of kefir.

- **Second Part: Shelf-life monitoring and regression analysis**

-Scatter plots were generated to visualize the evolution of quality parameters over time for both control and lemon-flavored kefir. Linear models were applied to each of the quality attributes, and coefficients, p-values, and adjusted R^2 values were examined, helping track quality changes over the storage period. By comparing regression models, we determined the points where quality parameters reached unacceptable levels, thereby establishing the shelf life of both kefir types. This analysis quantified the impact of lemon extract on kefir quality and preservation, highlighting potential benefits for shelf life extension.

- **Sensory analysis and consumer acceptance**

To assess consumer acceptance, sensory data were analysed and visualized using radar (spider web) graphs using the *fsmb* package. This provided an effective comparison of sensory profiles between the control and lemon-flavored kefir. In addition, analysis of variance was conducted on each of the sensory attributes.

- **Microbiological results conversion**

-For microbial counts, colonies within the range of 200–2000 were selected for each dilution.

-Concentrations were calculated using the formula:

$$\text{Concentration (CFU/g)} = \frac{\text{Number of colonies} \times \text{Homogenate} \times 10 \text{ Initial weight} \times \text{Aliquotize}}{\text{Weight of sample}}$$

LOG10 transformations of CFU/g concentrations were calculated, followed by averaging to determine microbiological values.

IV. Results and Discussion

The quality attributes of kefir are significantly influenced by the ratio of kefir grains to milk, as well as by the conditions of incubation, including time and temperature. In the first phase of this study, we systematically evaluated these variables to identify the optimal parameters that yield superior physicochemical, textural, and microbiological qualities in the final kefir product. Establishing these baseline conditions enabled us to proceed to the second phase, where we monitored the quality evolution of the fermented beverage over time to estimate its shelf life and assess the duration for which kefir retains its desirable properties.

This chapter presents a comprehensive analysis of the experimental findings for each quality parameter, contextualized through comparisons with existing studies conducted under similar objectives. By doing so, we aim to contribute valuable insights into the factors that influence kefir quality and longevity, further enhancing its value as a fermented dairy product.

Part 1: Establishing Optimal Fermentation Conditions

1. Physicochemical properties

The analysis revealed that all quality properties of kefir were influenced ($p < 0.05$) by the three primary factors, with first-order effects showing statistical significance in all cases. Additionally, two-way interactions between Ratio and Time, as well as between Time and Temperature, had a notable impact ($p < 0.05$) on pH and acidity. In contrast, certain quadratic terms for Time and Temperature also showed effects ($p < 0.05$) across all physicochemical properties except for syneresis (see Table 10). Table 10 presents an analysis of variance (ANOVA) for the response surface models fitted to the physicochemical parameters of kefir, specifically pH, acidity, and syneresis. The table outlines the mean square (Mean Sq) values and significance levels ($Pr(>F)$) for each parameter, highlighting the contributions of first-order (FO), two-way interaction (TWI), and quadratic terms to the model's fit.

➤ Key observations:

- **pH:** The pH model demonstrates strong statistical significance across several factors, with a particularly high mean square for the first-order term (FO), indicating it is significantly influenced by the primary factors ($p < 0.0001$). Both Ratio and Time ($p < 0.0001$) and Time and Temperature ($p = 0.003$) interactions show significant effects, while quadratic terms for Time and Temperature also show significant contributions ($p = 0.034$ and $p = 0.024$, respectively). The adjusted R^2 (R^2_{adj}) value of 0.913 and the

model's p-value ($p < 0.0001$) indicate a strong fit for the pH response model.

- **Acidity:** Acidity also shows strong dependence on first-order effects ($p < 0.0001$). The Time and Temperature interaction ($p = 0.0002$) significantly influences acidity, while the Ratio and Time interaction ($p = 0.003$) also contributes meaningfully. The adjusted R^2 for acidity is high (0.92), indicating that the model explains 92% of the variability in acidity, and the overall model significance ($p < 0.0001$) underscores its robustness.
 - **Syneresis:** The model for syneresis shows the least overall fit compared to pH and acidity, with an adjusted R^2 of 0.76. While significant first-order effects are observed ($p < 0.0001$), there are no significant two-way interactions or quadratic terms, suggesting a simpler relationship. The stationary points for syneresis ($R = 1.673$, $T = 8.9$, $\text{Time} = 35.7$) differ markedly from those of pH and acidity, which may reflect unique factors influencing this property.
- **Stationary points:** The stationary points for each parameter indicate the optimal combination of ratio (R), temperature (T), and time to achieve specific physicochemical targets:

For **pH:** $R = 0.48$, $T = 25.3^\circ\text{C}$, $\text{Time} = 24.2$ hours.

For **Acidity:** $R = 0.66$, $T = 25.3^\circ\text{C}$, $\text{Time} = 22.8$ hours.

For **Syneresis:** $R = 1.673$, $T = 8.9^\circ\text{C}$, $\text{Time} = 35.7$ hours.

This analysis highlights the importance of each factor and interaction term for optimizing kefir's physicochemical qualities, with a particularly strong fit for pH and acidity models, as shown by their high adjusted R^2 values and significant p-values.

Table 9: Analysis of variance of the response surface models fitted to the physicochemical parameters

	pH		Acidity		Syneresis	
	Mean Sq	Pr(>F)	Mean Sq	Pr(>F)	Mean Sq	Pr(>F)
FO	4.119	<0.0001	45.380	<0.0001	1785.58	<0.0001
TWI	-	-	-	-	-	-
Ratio.Time	0.905	<0.0001	5.445	0.003	-	-
Time.Temp	0.543	0.003	10.31	0.0002	-	-
Ratio.Temp	-	-	-	-	-	-
Ratio ²	-	-	-	-	-	-
Time ²	0.258	0.034	-	-	-	-
Temp ²	0.295	0.024	4.122	0.009	-	-
Residuals	0.05	-	0.495	-	61.53	-
Pure error	0.003	-	0.111	-	44.62	-
Stationary point	R=0.48 T=25.3 Time =24.2		R=0.66 T=25.3 Time =22.8		R=1.673 T=8.9 Time =35.7	
R ² adj	0.913		0.92		0.76	
P-value	<0.0001		<0.0001		<0.0001	

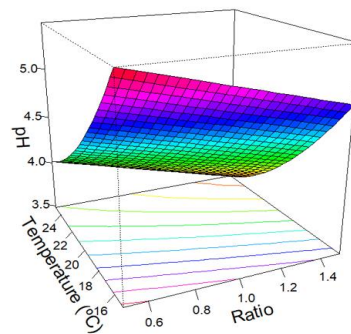
FO: Factorial Optimization, TWI: Two-Way Interaction, Pr: Probability, F: F-statistic, R²adj Adjusted R-squared, P value: Probability value

1.1.pH

The Figure 14 illustrates the response surface of pH in kefir in relation to varying temperature and kefir grains/milk ratio. The plot highlights the impact of these two factors on pH levels, showing how adjustments in grain concentration and temperature influence the acidity of the final product.

The pH of kefir reaches its maximum at a grains/milk ratio of 0.48%, a temperature of 25.3°C, and an incubation time of 24.2 hours (see Table 9). In this study, temperature and incubation time had minimal influence on pH variation, while using a lower concentration of kefir grains resulted in the highest pH values (>4.5) (see Fig. 3).

Consistent with these findings, Dewi et al. (2020) reported that the highest pH in goat's milk kefir was observed when using the lowest concentration of kefir grains without storage, while the lowest pH occurred in samples made with the highest concentration of kefir grains after 21 days of storage. This suggests that the concentration of kefir grains and the duration of storage significantly affect the acidity of goat's milk kefir over time, with higher grain concentrations and extended storage periods associated with increased acidity.



Slice at FermentationTime = 20

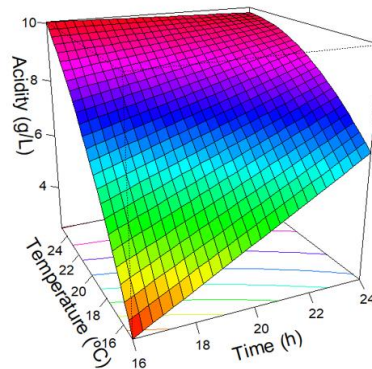
Figure 3: Response surface of pH as a function of temperature and grains/milk ratio

1.2.Acidity

The acidity of kefir is maximized at a grains/milk ratio of 0.66%, a temperature of 25.3°C, and an incubation time of 22.8 hours (see Table 9). Among the three factors, temperature had the most substantial influence on acidity, followed by incubation time, with the grains/milk ratio exerting the least effect (see Fig. 4).

Aligned with these findings, Putri, Setiani, and Warya (2020) reported that optimization of incubation time and temperature was crucial to maximize lactic acid production in kefir fermentation with the addition of a starter culture. Organic acid levels increased with fermentation time at both room temperature and higher temperatures.

Prolonged fermentation periods allowed greater substrate conversion by the ferment, resulting in higher organic acid concentrations. Warmer temperatures proved optimal for kefir fermentation due to increased organic acid production. The study concludes that temperature and incubation time significantly influence organic acid levels, with higher temperatures favoring optimal growth of lactic acid bacteria and longer fermentation times enabling increased lactic acid production from milk lactose.

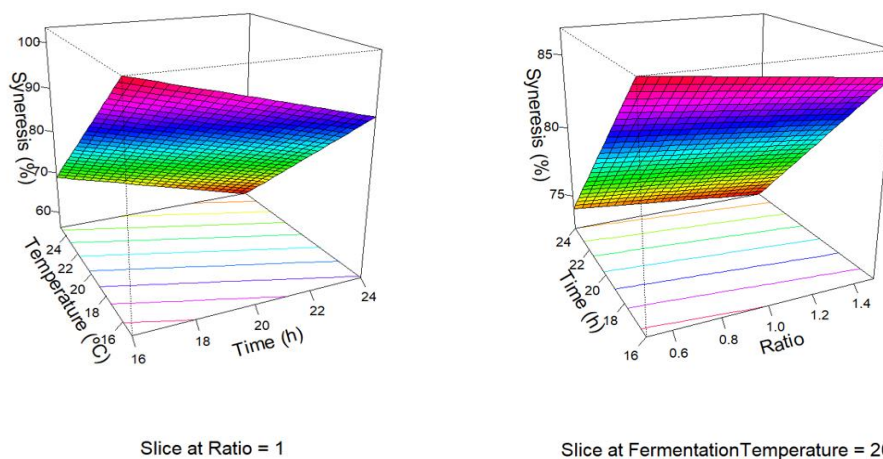


Slice at Ratio = 1

Figure 4: Response surface of acidity as a function of temperature and time of incubation

1.3.Syneresis

Lower temperatures below 15°C and shorter incubation times under 16 hours both lead to increased syneresis levels, exceeding 90%, demonstrating an inverse relationship between these factors and syneresis (see Table 10). Conversely, as the grains/milk ratio increases, there is a weak negative effect on syneresis, resulting in a slight reduction. In other words, higher grain/milk ratios correspond to a minor decrease in syneresis levels (see Fig. 5).



Slice at Ratio = 1

Slice at FermentationTemperature = 20

Figure 5: Response surface of syneresis

At an incubation temperature of 25°C, the syneresis of goat's milk kefir ranged from 38.92% to 61.47%, whereas at the lowest incubation temperature of 15°C, high syneresis levels were observed, reaching between 93.97% and 95.54% (see Table 10).

- High syneresis: Runs with a lower temperature (15-20°C) and shorter time (16 hours) tend to have a higher mean syneresis percentage, such as Run 1 (96.41%) and Run 5 (95.54%).
- Lower syneresis: Some runs, like Run 10, have notably lower syneresis (6.026%) under conditions of ratio 1.5, time 16 hours, and temperature 20°C.
- Variability: The standard deviation (sd) values indicate how much variation there is within each set of conditions. For example, Run 7 (81.19 ± 14.9) shows substantial variability, suggesting inconsistent syneresis under those conditions.
- Effect of ratio: Increasing the ratio (e.g., from 0.5 to 1 or 1.5) appears to reduce syneresis in some instances, although this effect is inconsistent and likely depends on the specific combination of time and temperature.

Table 10: Summary statistics of the syneresis of kefir treatments

Run#	Ratio	Time	Temperature	Syneresis (mean \pm sd)
1	0.5	16	20	96.41 \pm 0.5586
2	0.5	20	15	95.51 \pm 0.6258
3	0.5	20	25	38.92 \pm 0.9546
4	0.5	24	20	61.08 \pm 0.2104
5	1	16	15	95.54 \pm 0.4419
6	1	16	25	60.31 \pm 0.1927
7	1	20	20	81.19 \pm 14.9
8	1	24	15	95.18 \pm 0.4419
9	1	24	25	61.47 \pm 0.6346
10	1.5	16	20	6.026 \pm 0.5144
11	1.5	20	15	93.97 \pm 0.1998
12	1.5	20	25	60.03 \pm 0.4844
13	1.5	24	20	68.88 \pm 0.7814

2. Textural properties

The results indicate that the two-way interaction between **Time** and **Temperature** significantly influenced the textural characteristics of kefir, specifically **firmness**, **consistency**, and **cohesiveness** ($p < 0.05$). These findings underscore the critical role of processing conditions, where variations in incubation time and temperature combinations can lead to notable alterations in the physical structure and sensory qualities of kefir. Such interactions suggest that kefir's microstructure may respond sensitively to thermal exposure over time, likely impacting protein gel network formation and moisture retention, which are essential determinants of its overall textural integrity. Furthermore, an analysis of **quadratic terms** highlighted the significant effect of **Ratio** and **Temperature** ($p < 0.05$) across nearly all evaluated textural properties, except for the **viscosity index** (Table 11).

This non-linear relationship implies that slight modifications in ingredient proportions (Ratio) and temperature yield compounded effects on texture, indicating an optimal threshold within these parameters. However, the viscosity index's lack of sensitivity to quadratic effects suggests

a more stable response, potentially due to its dependence on intrinsic viscosity modifiers that are less affected by ingredient concentration or thermal conditions.

The significance of these quadratic effects reveals a complex, non-linear interplay between compositional and processing factors, emphasizing the need for precise control over formulation and incubation parameters to achieve desirable textural outcomes in kefir production. These insights align with previous findings that highlight the importance of multi-factorial interactions in fermented dairy products, reinforcing the role of controlled processing for targeted textural and sensory attributes.

The analysis of variance (ANOVA) for the response surface models (Table 11) reveals significant effects of specific factors and interactions on the textural parameters of kefir, with model terms showing statistical significance at varying levels. The parameters assessed include **Firmness, Consistency, Cohesiveness**, and the **Index of Viscosity**.

- **Firmness:** For **firmness**, the significant model terms include **Time and Temperature interaction (Time.Temp)** and **Temperature squared (Temp²)**, both with p-values below 0.05 (0.002 and 0.011, respectively). These findings indicate that the combination of time and temperature, along with non-linear effects of temperature, plays a pivotal role in determining firmness in kefir. Notably, the **stationary point** for optimal firmness occurs at a **Ratio of 1.07, Temperature of 19.9°C, and Time of 17.4 hours**, suggesting that these conditions yield the most desirable firmness outcome.

- **Consistency:** The **consistency** of kefir is significantly influenced by multiple factors, including **Time.Temp** and **Temp²**, with p-values of 0.002 and 0.018, respectively. Additionally, the **two-way interaction between Ratio and Temperature (Ratio.Temp)** is significant with a p-value of 0.025, underscoring the complex interplay of compositional and thermal factors on consistency. The stationary point for optimal consistency aligns closely with that of firmness, occurring at **Ratio 1.07, Temperature 19.9°C, and Time 17.4 hours**. The consistency model demonstrated a high degree of fit, with an **adjusted R² value of 0.626** and an overall model p-value of <0.0001, confirming model robustness.

- **Cohesiveness:** In the **cohesiveness** model, significant terms include **Time.Temp** and **Ratio²**, both with a p-value of 0.001. Additionally, **Temp²** also has a notable impact (p=0.010), indicating that cohesiveness is sensitive to both linear and quadratic variations in temperature and ratio. The stationary point for cohesiveness occurs at a **Ratio of 1.16, Temperature of 22.6°C, and Time of 25.1 hours**, suggesting a higher temperature and longer processing time for optimal cohesiveness. With an **adjusted R² of 0.609** and a model p-value of 0.0001, the

cohesiveness model is statistically robust, capturing substantial variation in response.

- Index of Viscosity: For the **index of viscosity**, however, no significant effects are observed across the tested variables or their interactions (all terms have non-significant p-values). The absence of significance here suggests that viscosity may be relatively stable or unaffected by the parameters studied. This is further supported by a comparatively lower adjusted R^2 of 0.5202, indicating the model explains a modest amount of variance in the index of viscosity. The models for **firmness, consistency, and cohesiveness** demonstrate strong statistical significance and high levels of fit, as indicated by adjusted R^2 values of 0.628, 0.626, and 0.609, respectively, and overall p-values of <0.0001 . These findings validate the reliability of the response surface models for these textural parameters. However, the model for the index of viscosity shows limited predictive capacity, reflected in its adjusted R^2 of 0.5202 and an overall p-value of 0.001. These insights confirm that time, temperature, and ingredient ratios are influential factors in defining the structural and sensory properties of kefir, with significant non-linear effects on firmness, consistency, and cohesiveness. In contrast, the index of viscosity remains relatively unaffected, indicating that viscosity in kefir may be governed by other intrinsic properties beyond those studied.

Table 11: Analysis of variance of the response surface models fitted to the textural parameters

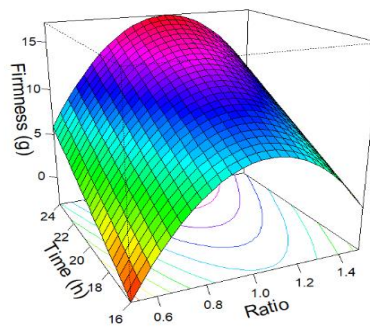
	Firmness		Consistency		Cohesiveness		Index of viscosity	
	Mean Sq	Pr(>F)	Mean Sq	Pr(>F)	Mean Sq	Pr(>F)	Mean Sq	Pr(>F)
FO	277.90	0.005	21613	0.006	15.526	0.017	1.154	0.004
TWI	-	-	-	0.025	-	-	-	-
Ratio.Time	-	-	-	-	-	-	-	-
Time.Temp	592.20	0.002	44104	0.002	29.280	0.010	-	-
Ratio.Temp	-	-	-	-	-	-	-	-
Ratio ²	679.26	0.001	50182	0.001	51.700	0.001	-	-
Time ²	-	-	-	-	-	-	-	-
Temp ²	368.17	0.011	23690	0.018	49.187	0.001	-	-
Residuals	-	-	3573	-	3.681	-	0.2	-
Pure error	-	-	188	-	0.948	-	0.13	-
Stationary point	R=1.07 Time =17.4	T=19.9	R=1.07 Time =17.4	T=19.9	R=1.16 Time =25.1	T=22.6	R=1.28 Time =20.4	T=18.5
R ² adj	0.628		0.626		0.609		0.5202	
P-value	<0.0001		<0.0001		0.0001		0.001	

FO: Factorial Optimization, TWI: Two-Way Interaction, Pr: Probability, F: F-statistic, R²adj Adjusted R-squared, P value: Probability value

2.1. Firmness

The firmness of kefir is notably affected by incubation conditions, particularly **incubation time**, **temperature**, and the **grain/milk ratio**. As Table 11 demonstrates, each of these variables plays a significant role in determining firmness ($p < 0.05$). The **grain/milk ratio**, a critical factor in fermentation, exerts a complex influence on firmness, with both extremely low ($<0.6\%$) and high ($>1.6\%$) ratios leading to a marked reduction in firmness, falling below 5g. This non-linear relationship, as shown in Figure 6, suggests that a balanced ratio is essential to maintain the desired structural integrity of the kefir, likely due to the optimal balance between fermentation agents and available nutrients within the milk matrix.

Further contributing to this relationship, the **quadratic term of temperature** is significant, indicating that firmness responds not only to temperature in a linear fashion but also to its squared effect. This quadratic influence implies that small changes in temperature near the optimal range can either enhance or weaken firmness, depending on the direction of deviation. Such sensitivity to temperature adjustments may stem from alterations in the gel structure formed during fermentation, where moderate thermal conditions support a stable protein network, while extremes may disrupt it. The interactive effects of incubation parameters highlighted in this model underscore the necessity of precise control over these variables in kefir production. Optimal settings for firmness can thus be achieved through careful calibration of grain/milk ratios and thermal conditions, as well as the duration of incubation. These findings are crucial for developing standardized processing protocols to ensure consistent quality in kefir texture.



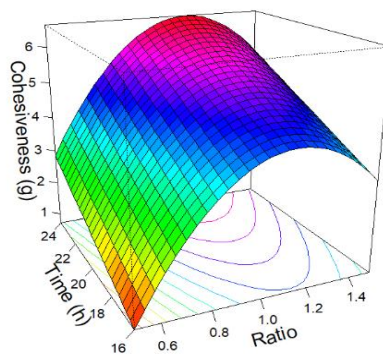
Slice at FermentationTemperature = 20

Figure 6: Response surface of firmness as a function of time and grains/milk ratio

2.2. Cohesiveness

The **cohesiveness** of kefir, a key textural attribute affecting its sensory appeal and mouthfeel, is significantly impacted by **incubation duration**, **temperature**, and the **grain/milk ratio**. Statistical analysis in Table 11 indicates that these factors interact to influence cohesiveness,

with p-values indicating their relevance ($p < 0.05$). Cohesiveness exhibits a pronounced sensitivity to the grain/milk ratio, as reductions below 3g are observed at both low (<0.6%) and high (>1.4%) ratios, as depicted in Figure 7. These extremes likely disturb the delicate balance of microbial activity and nutrient availability, impairing the formation of a cohesive protein matrix. Moreover, the quadratic term of temperature demonstrates significant modulation of cohesiveness under high grain/milk ratios, emphasizing the non-linear nature of temperature's effect in these conditions. This quadratic relationship suggests that slight deviations from optimal temperature settings in either direction can further reduce cohesiveness when the grain/milk ratio is high, possibly due to disruption in the gel network structure. Under these conditions, temperature sensitivity may relate to changes in protein interaction and water retention, both of which are critical to maintaining cohesive consistency in fermented dairy matrices. The findings highlight the importance of balancing **grain/milk ratios** and maintaining controlled thermal conditions to achieve optimal cohesiveness. By fine-tuning these parameters, production protocols can be developed to enhance the uniformity and quality of kefir's texture, ensuring a consistent and desirable product for consumers. This nuanced understanding of the interactions between incubation variables provides a basis for refining kefir fermentation processes.



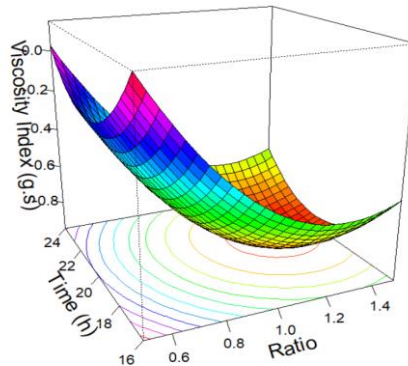
Slice at Fermentation Temperature = 20

Figure 7: Response surface of cohesiveness as a function of time and grains/milk ratio

2.3. Viscosity index

The viscosity index of kefir, an essential parameter reflecting its thickness and flow properties, is significantly influenced by both incubation time and temperature, as well as the grain/milk ratio. Analysis in Table 11 shows that the combination of a higher grain/milk ratio (>1%) and prolonged incubation periods (>20 hours) results in the highest recorded viscosity index values (>1 g·s), as illustrated in Figure 8. This trend suggests that extended fermentation time and elevated grain content enhance the microbial activity and metabolic processes,

leading to a denser, more viscous texture. Such conditions may promote increased production of exopolysaccharides and denser protein network formation, which contribute to the viscosity of the final product.



Slice at Fermentation Temperature = 20

Figure 8: Response surface of viscosity as a function of time and grains/milk ratio

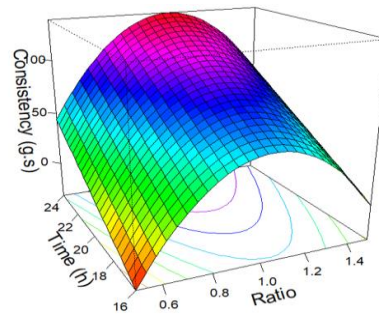
To minimize the viscosity index in kefir, it is essential to maintain a **grain/milk ratio below 1%** and limit the **incubation time to less than 22 hours**. These adjustments can prevent excessive thickening, yielding a smoother and more fluid product, which may be desirable depending on consumer preferences and product specifications.

Interestingly, these findings differ from those of Dewi et al. (2020), who observed an increase in the viscosity of goat's milk kefir with a higher concentration of kefir grains. This divergence may be attributable to differences in milk composition between goat and cow milk, as well as potential variations in microbial populations and activity levels in different milk types. Goat milk's unique protein and fat profile may respond differently to high kefir grain concentrations, resulting in higher viscosity compared to cow milk kefir under similar conditions. The current findings underscore the importance of tailoring incubation parameters and grain/milk ratios in kefir production to achieve specific textural and viscosity outcomes. Understanding these variable effects offers a framework for controlling viscosity in kefir, enabling producers to meet diverse consumer demands while maintaining product quality and consistency.

2.4. Consistency

The **consistency** of kefir, which reflects its textural stability and uniformity, is significantly affected by **incubation duration, temperature, and the grain/milk ratio**. Statistical analysis in Table 11 reveals that these factors exert a substantial impact on consistency, with p-values indicating their significance ($p < 0.05$). The optimal consistency value, exceeding **100 g·s**, was achieved at a **1% grain/milk ratio** combined with an extended **24-hour incubation period**.

These conditions likely create an ideal balance, enabling optimal microbial fermentation and nutrient interaction to produce a cohesive and stable kefir matrix.



Slice at FermentationTemperature = 20

Figure 9: Response surface of consistency as a function of time and grains/milk ratio

However, consistency shows a marked decrease (<50 g·s) at both extreme grain/milk ratios, specifically below 0.6% and above 1.4%, as demonstrated in Figure 9. These reductions highlight the sensitivity of kefir's texture to the grain/milk ratio, as both insufficient and excessive grain content can disrupt the fermentation process. Low ratios may limit microbial activity and structural gel formation, while high ratios could lead to overcrowding and competition among microbes, negatively impacting texture.

Furthermore, the **quadratic temperature term** shows statistical significance under high-ratio conditions, underscoring the non-linear effect of temperature at elevated grain/milk ratios. This quadratic relationship suggests that slight increases or decreases in temperature under high grain content conditions can either stabilize or destabilize the kefir consistency. This may result from temperature-dependent changes in microbial growth rates and fermentation kinetics, which in turn affect protein interactions and the consistency of the kefir. These findings underscore the importance of controlling incubation parameters to achieve optimal consistency in kefir. Balancing the grain/milk ratio, along with careful modulation of time and temperature, is critical for producing a product with a stable and desirable texture. This knowledge is valuable for standardizing production practices and aligning kefir texture with consumer expectations.

3. Microbiological properties

The microbiological characteristics of kefir, particularly the populations of **lactic acid bacteria (LAB)** and **mesophiles (MES)**, are significantly influenced by the incubation parameters of temperature, time, and grain-to-milk ratio. Statistical analysis, as presented in Table 12, highlights the effects of these variables on microbial activity, which is critical for kefir's fermentation quality and its health benefits. The **quadratic temperature term** demonstrated statistical significance across all microbiological properties ($p < 0.05$), indicating that

temperature plays a non-linear role in modulating microbial growth. Specifically, the effect of temperature on both **lactic acid bacteria** and **mesophiles** reflects its pivotal role in controlling metabolic activity and growth rates within the microbial community. This quadratic relationship suggests that optimal growth conditions occur within a certain temperature range, while deviations above or below this range can diminish microbial viability or metabolic efficiency. For LAB, the stationary point was observed at a Temperature of 21.9°C and an incubation Time of 21.2 hours, while for MES, the optimal temperature was slightly lower, at 21.4°C, with a longer Time of 23.2 hours. These temperatures align closely with the optimal conditions traditionally reported for LAB, reinforcing their importance in achieving a balanced microbial environment within kefir. Unlike temperature, the grain/milk ratio demonstrated a significant effect only on mesophilic populations ($p = 0.011$). This selective influence indicates that mesophiles are particularly sensitive to variations in grain concentration, which may alter the availability of nutrients and fermentation substrates. The optimal grain/milk ratio for mesophilic growth was identified at **0.61**, a moderate concentration that likely supports adequate microbial competition and nutrient access. Excessive grain content, however, may disrupt mesophilic stability by creating excessive microbial density, thus impacting the overall balance of the kefir culture.

Table 12: Analysis of variance of the response surface models fitted to the microbiological parameters

	Mesophiles			Lactic acid bacteria		
	Mean Sq	Pr(>F)		Mean Sq	Pr(>F)	
FO	6.960	<0.0001		5.452	<0.0001	
TWI	-	-		-	-	
Ratio ²	4.368	0.011		-	-	
Time ²	-	-		-	-	
Temp ²	2.588	0.049		1.787	0.052	
Residuals	0.573	-		0.428	-	
Pure error	0.227	-		0.222	-	
Stationary point	R=0.61	T=21.4	Time =23.2	R=0.33	T=21.9	Time =21.2
R ² adj			0.617			0.587
P-value			<0.0001			<0.0001

FO: Factorial Optimizatiion. TWI: Two-Wav Interaction. Pr: Probabilitv. F: F-statistic. R²adi Adjusted R-squared. P value: Probabilitv value

The response surface models for both microbial parameters exhibit strong fit, with **adjusted R² values** of 0.617 for MES and 0.587 for LAB, and overall model p-values of <0.0001, underscoring the models' robustness. These findings highlight the importance of precise control over temperature and grain/milk ratios to optimize microbial populations, particularly for achieving a beneficial balance of LAB and mesophiles. Optimizing these microbial populations

is essential for ensuring the fermentation quality, texture, and potential probiotic benefits of kefir. In conclusion, these results demonstrate that achieving the optimal microbiological profile in kefir requires careful calibration of temperature and grain/milk ratios. This study provides a foundation for standardized protocols in kefir production, supporting both the safety and probiotic potential of the final product.

3.1. Lactic acid bacteria

The concentration of **lactic acid bacteria (LAB)** in kefir, a crucial determinant of its probiotic qualities and fermentation efficiency, is significantly influenced by the **grain/milk ratio**. Statistical analysis in Table 12 and Figure 10 highlights a strong positive correlation between LAB concentration and the grain/milk ratio, suggesting that increasing grain content directly supports LAB proliferation. This relationship likely arises due to the higher microbial load and availability of nutrients associated with increased grain content, which enhances LAB growth and activity within the kefir matrix. While **incubation time** also affects LAB concentration, its influence is comparatively weaker than that of the grain/milk ratio. The relatively modest effect of time suggests that LAB populations reach a growth plateau relatively early in the fermentation process, indicating that extended incubation may not significantly boost LAB concentration once optimal growth conditions are met. The analysis reveals a complex, non-linear relationship between temperature and LAB concentration, as evidenced by the statistical significance of the quadratic temperature term ($p < 0.05$). This finding implies that the effect of temperature on LAB concentration is not uniform across all ranges; rather, specific temperature thresholds likely promote optimal growth while deviations (either above or below) may reduce microbial activity. Such a non-linear response may be due to temperature-sensitive metabolic pathways in LAB, where mild increases near the optimal range enhance enzymatic activity and growth, while extreme temperatures inhibit these processes. This sensitivity to temperature underscores the importance of precise thermal control in optimizing LAB concentration during kefir fermentation. Interestingly, neither the quadratic term for the grain/milk ratio nor that for incubation time showed statistical significance, indicating that these factors influence LAB concentration in a largely linear manner within the tested ranges. This suggests that LAB responds predictably to adjustments in the grain/milk ratio and time, with no evidence of complex threshold effects for these variables within the observed data. These findings underscore the critical role of temperature and grain/milk ratio in determining LAB concentration in kefir. To achieve high LAB concentrations and, consequently, a robust probiotic profile, it is essential to maintain the grain/milk ratio at a level that supports optimal microbial growth while also closely monitoring temperature to avoid thresholds that may

negatively impact LAB populations. By balancing these factors, kefir producers can create a product that meets both quality and probiotic standards, ensuring consistency in microbial content and health benefits for consumers.

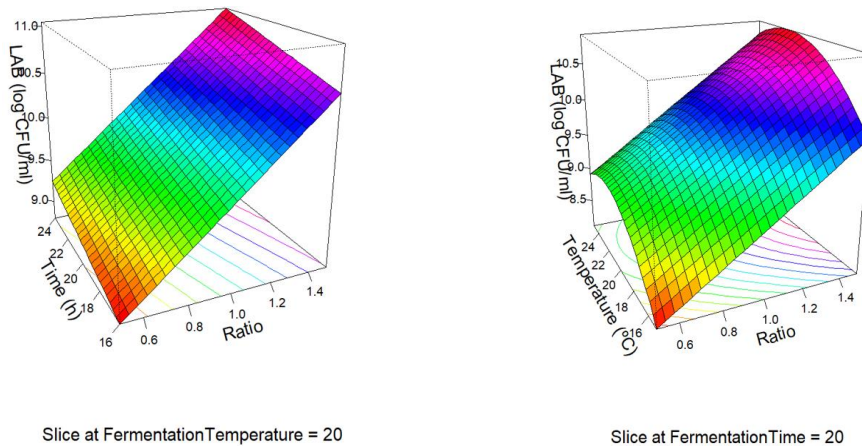


Figure 10: Response surface of lactic acid bacteria

3.2. Mesophiles

The concentration of **mesophilic bacteria** in kefir is predominantly affected by the **grain/milk ratio**, as illustrated in Figure 11. This strong association suggests that increasing the grain/milk ratio supports mesophilic growth by enhancing nutrient availability and microbial load, fostering an environment conducive to the proliferation of these bacteria. In comparison, **incubation time and temperature** exert a relatively minor influence on mesophilic concentration, indicating that the growth dynamics of mesophiles are less sensitive to time and temperature adjustments within the tested range. Further analysis, as shown in Table 12, reveals that the **quadratic terms for both temperature and grain/milk ratio** are statistically significant ($p < 0.05$) in relation to mesophilic concentration. This significance indicates that the effects of these variables on mesophilic populations follow a **curvilinear or parabolic pattern**, rather than a straightforward linear relationship. Such a relationship suggests that as temperature and grain/milk ratio increase, mesophilic growth reaches an optimal point before declining or stabilizing, forming a U-shaped or bell-shaped curve.

This non-linear relationship implies that mesophilic concentration responds dynamically to temperature and grain/milk adjustments, where minor deviations from the optimal range can result in substantial variations in bacterial growth. For instance, at temperatures slightly below or above the ideal range, the metabolic efficiency of mesophilic bacteria may decrease, inhibiting growth or survival. Similarly, grain/milk ratios that are too high or too low may disrupt microbial balance or nutrient accessibility, thus limiting mesophilic activity.

The identification of these non-linear effects is crucial for optimizing kefir fermentation. By maintaining temperature and grain/milk ratio within specific ranges, kefir producers can maximize mesophilic concentration, ensuring product consistency and quality. The curvilinear response of mesophiles suggests that precise control of fermentation conditions is necessary to avoid suboptimal growth conditions, which could lead to inconsistencies in microbial concentration and, consequently, in the sensory and health properties of the final product.

In conclusion, the **grain/milk ratio** is the primary factor influencing mesophilic concentration, but the **temperature and grain/milk interactions** exhibit non-linear dynamics. These findings provide valuable insights for developing controlled, reproducible fermentation processes that support stable mesophilic populations in kefir.

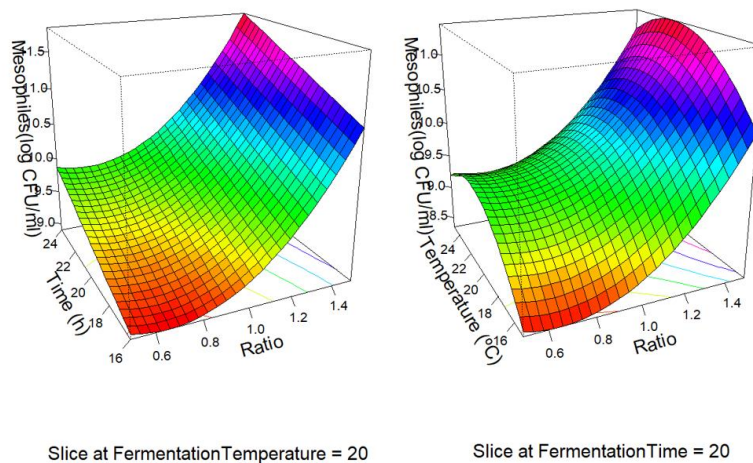


Figure 11: Response surface of mesophiles

3.3. Yeasts and molds

The statistical analysis of **yeast and mold populations** in the kefir samples indicated a lack of significant results for these microbial groups. As summarized in Table 13, the experimental parameters—such as incubation time, temperature, and grain/milk ratio—did not show a statistically significant impact on the concentrations of yeasts and molds. This outcome limited the ability to develop a reliable **response surface model**, as the observed variability in yeast and mold populations could not be sufficiently explained by the factors tested.

The absence of statistically significant effects suggests that the selected incubation parameters may not exert substantial influence over yeast and mold growth within the experimental range. This finding implies that these microbial populations are potentially influenced by other factors not included in the current experimental design or are more resilient to changes in the tested variables. Alternatively, it is possible that the optimal growth conditions for yeasts and molds are outside the range of the experimental conditions applied, leading to limited variation and, consequently, to non-significant results. This outcome highlights the need for additional research into the factors that may more meaningfully influence yeast and mold

populations in kefir, such as variations in pH, oxygen availability, or specific nutrient concentrations. Identifying the correct environmental or compositional factors for modeling yeast and mold behavior could provide deeper insights into the fermentation ecology of kefir and support more comprehensive microbial management in production settings. In conclusion, the non-significance of the parameters for yeast and mold growth underlines the complexity of kefir's microbial ecosystem. Future studies may consider a broader range of variables to clarify the conditions that favor or inhibit these populations, ensuring a more complete understanding of kefir's microbial composition and potential impacts on product quality.

- **Yeast populations:**

The yeast concentrations exhibit low and generally negative log CFU/ml values for most runs, indicated by a mean of **-0.301 log CFU/ml** in many entries, with little or no variation (sd = 0). Positive yeast counts are sporadically observed, with only a few runs (such as Run 2, Run 5, Run 8, Run 10 and Run 11) showing positive values above 0 log CFU/ml. The highest recorded mean yeast concentration is 1.142 log CFU/ml in Run 8 (Ratio = 1, Time = 24 hours, Temperature = 15°C), suggesting that under this combination of conditions, yeast populations showed some increase. However, variability remains minimal, and many standard deviations are zero, indicating that yeast growth did not significantly fluctuate under different conditions.

- **Mold populations:**

Mold concentrations generally follow a similar pattern to yeasts, with most runs showing concentrations around **-0.301 log CFU/ml**, concentrations around indicating low to negligible mold counts across the treatments. A few positive mold concentrations were recorded, such as in Run 1 and Run 3, with Run 12 showing a slightly higher mean mold concentration of **0.8495 log CFU/ml** (Ratio = 1.5, Time = 20 hours, Temperature = 25°C). Despite this, molds generally exhibit low variability and do not respond significantly to the treatment conditions.

- **Implications of findings:**

The predominantly negative or zero values for both yeasts and molds suggest minimal growth under the tested experimental conditions, corroborating previous observations that incubation time, temperature, and grain/milk ratio have limited influence on these microbial groups.

The sporadic positive values indicate that under certain conditions (e.g., higher ratio and prolonged time), yeasts and molds may exhibit slight growth; however, these conditions do not produce substantial or consistent increases.

The data further supports the conclusion that **yeasts and molds** are relatively insensitive to the experimental variables within the tested ranges, indicating that other factors—possibly external

to this study—are likely needed to significantly influence yeast and mold populations in kefir. Table 13 illustrates that yeasts and molds remain largely unaffected by the controlled variables of ratio, time, and temperature, with minor variations insufficient to build a reliable predictive model. This reinforces the need for exploring additional parameters to understand the behavior of yeasts and molds in kefir fermentation.

Table 13: Summary statistics of the microbiological characteristics (log CFU/ml) of kefir treatments

Run#	Ratio	Time	Temperature	Yeasts (mean±sd)	Molds(mean±sd)
1	0.5	16	20	-0.301 ± 0.01	0.0242 ± 0.460
2	0.5	20	15	0.349 ± 0.920	-0.301 ± 0.01
3	0.5	20	25	-0.301 ± 0.01	0.0242 ± 0.460
4	0.5	24	20	-0.301 ± 0.01	-0.301 ± 0.01
5	1	16	15	0.369 ± 0.027	-0.301 ± 0.01
6	1	16	25	-0.301 ± 0.01	-0.301 ± 0.01
7	1	20	20	-0.301 ± 0.01	-0.301 ± 0.02
8	1	24	15	1.142 ± 0.017	-0.2258 ± 0.106
9	1	24	25	-0.301 ± 0.01	-0.301 ± 0.01
10	1.5	16	20	0.349 ± 0.01	-0.301 ± 0.01
11	1.5	20	15	0.250 ± 0.072	-0.301 ± 0.01
12	1.5	20	25	-0.301 ± 0.01	0.8495 ± 0.01
13	1.5	24	20	-0.301 ± 0.01	-0.301 ± 0.01

→ In this phase of the research, we successfully achieved targeted improvements in **kefir's thickness and flow properties**, optimizing its textural and sensory qualities to enhance consumer appeal. By adjusting incubation conditions, we determined that kefir with the **desired acidity, firmness, and consistency** can be produced at **lower incubation temperatures (15 - 20°C)** and **extended incubation times (20 - 24 hours)**, provided the **grains/milk ratio is increased to levels above 1.0%**. This combination promotes an optimal balance of microbial activity and texture formation, resulting in a kefir product with improved flow characteristics while maintaining the integrity of its acidic profile.

Based on these findings, the upcoming phase of research — **Extending the Shelf-life** — will employ a **grains/milk ratio of 0.9%**, an **incubation time of 24 hours**, and an **incubation temperature of 20°C**. This parameter combination aims to sustain the desired textural qualities over an extended storage period, focusing on microbial stability and product longevity.

Part 2: Assessing and Enhancing the Shelf-Life of Kefir

This section presents a comprehensive comparison between two kefir varieties: a **control sample** and a **lemon-flavored variant** enhanced with lemon extract. Our analysis encompasses a broad range of quality attributes essential to understanding kefir's product integrity, consumer appeal, and shelf stability. Specifically, we will evaluate **physicochemical properties** (such as pH and acidity), **textural characteristics** (including viscosity and consistency),

microbiological profiles (focusing on lactic acid bacteria and mesophiles counts), and **sensory attributes** (assessing taste, odor, and overall palatability).

Through this multifaceted approach, we aim to:

1. **Determine the shelf life** of the two kefir varieties under standardized storage conditions.
2. **Assess the effect of lemon extract** on extending product shelf life by potentially influencing microbial stability and physicochemical properties.
3. **Evaluate consumer acceptance and preference** for each variety, exploring the potential for lemon flavoring to enhance kefir's market appeal.

This in-depth study will provide valuable insights into the benefits of incorporating natural additives, such as lemon extract, in kefir production. By examining the impact of flavoring on both product longevity and consumer enjoyment, this research contributes to a broader understanding of natural flavour additives as viable tools for improving kefir quality, extending shelf life and enhancing product marketability. The results will offer key considerations for kefir producers aiming to expand their product range and meet evolving consumer preferences in the dairy industry.

4. Physicochemical properties

An in-depth analysis of **physicochemical properties** for control and lemon extract-flavored kefir was conducted over a 12-day storage period. Using **scatter plots generated in RStudio**, we visualized the distinct trends in physicochemical evolution between the two kefir varieties, with the graphical data highlighting notable differences in key parameters such as **pH, titratable acidity, syneresis, and proteolysis**.

The scatter plots reveal significant divergence between the two products over time. For instance, pH levels and titratable acidity—indicators of fermentation and microbial activity—displayed distinct trends between the control and lemon-flavored samples, suggesting that the **addition of lemon extract impacts acidity levels** and potentially moderates the natural fermentation process. Syneresis and proteolysis trends similarly varied, with the flavored kefir demonstrating different rates of liquid separation and protein breakdown, indicating a possible effect of lemon extract on kefir's structural stability and texture. This graphical representation not only facilitated the identification of differences in the physicochemical profiles of the two kefir varieties but also provided **valuable insights into the impact of lemon extract on product stability and shelf life**.

By quantifying the observed variations in these scatter plots, we established a solid basis for evaluating the **efficacy of lemon extract as a natural preservative** and its

influence on key physicochemical attributes throughout kefir's shelf life. The findings thus contribute to a deeper understanding of how natural flavor additives, like lemon extract, may affect both the stability and sensory quality of fermented dairy products over time.

4.1. pH

The analysis of variance (ANOVA) and least square means (Table 14) indicate that **no significant interaction** ($p > 0.05$) was observed between storage duration and treatment type (control vs. lemon-flavored kefir) in relation to **pH** levels. This finding contrasts with the study by Dewi et al. (2020), which identified a significant interaction between storage time and grain/milk ratio in goat's milk kefir, showing a pronounced effect on pH evolution. In our study, **storage time alone had a highly significant impact on pH** ($p < 0.01$), indicating that the duration of storage is a critical determinant of pH levels in both control and -flavored kefir.

From the scatter plot (Fig. 12), we observe that on the first day of fermentation (**Day 0**), the initial pH of the lemon-flavored kefir was **4.12**, slightly lower than the control kefir's pH of **4.25** due to the acidic influence of lemon extract. During the first three days of storage, both kefir varieties exhibited a **significant increase in pH** ($p < 0.5$), followed by a gradual decline until **Day 6**. The effect of storage duration on pH was particularly pronounced, with flavored kefir reaching its peak pH of **4.4 on Day 9**, after which the pH steadily declined, reaching **4.0 by Day 12**. By contrast, the control kefir displayed relative stability in pH, with minor fluctuations and a final pH of **4.28** at the end of the storage period, aligning with the slight mid-storage drop in pH seen in both varieties.

This trend aligns closely with findings from Akan (2020) and Solanki, Ghosh, and Kumawat (2023), who reported a similar pattern in control kefir, where an initial pH of **4.47** declined to **4.37** on Day 6 before increasing to **4.46** by Day 12. Similar trends in pH evolution have been observed by Putri, Setiani, and Warya (2020) in goat's milk kefir, with an increase from **3.62 to 4.54** over a 12-day cold storage period (6–10°C), reinforcing the general trend of rising pH during extended storage. These studies suggest that temperature and storage conditions influence pH changes, although specific values may vary with milk type and exact storage conditions.

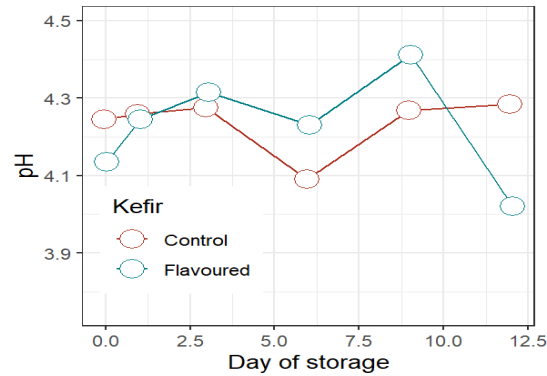


Figure 12: Evolution of kefir pH during shelf life

Interestingly, while most studies point to a gradual pH increase over storage time, a few, such as Irigoyen et al. (2005), reported no significant pH changes during storage. This discrepancy may reflect differences in kefir formulations, microbial compositions, and environmental conditions, which can alter the metabolic activity within kefir cultures and, consequently, pH evolution over time.

Our study demonstrates that **lemon extract enhances initial acidity**, yet both control and flavored kefir follow a comparable pattern of pH fluctuation over a 12-day storage period, with a significant mid-period drop and a gradual return to higher pH levels. These findings contribute to a nuanced understanding of how storage duration and flavoring agents like lemon extract can influence the acidity profile and shelf stability of kefir products, offering valuable insights for optimized product formulation.

4.2. Acidity

Acidity in kefir is primarily a result of **lactic acid fermentation**, where lactic acid bacteria (LAB) metabolize lactose, producing lactic acid as a by-product. This acidification process is crucial for kefir's sensory qualities and microbial stability. According to the **Codex Alimentarius** standard for fermented milks, a minimum lactic acid content of **0.6%** is required for kefir, establishing a benchmark for its essential composition and quality (Codex Alimentarius, 2022)

In this study, **ANOVA results** (Table 14) reveal that the **treatment type** (control vs. lemon-flavored) significantly influenced acidity values in both kefir varieties, with a p-value of **<0.05**. The presence of lemon extract in the flavored kefir contributed to an initial increase in acidity, which not only aligns with its sensory profile but also enhances the microbial preservation effect. Furthermore, both **storage duration** and the **interaction between storage time and treatment** significantly impacted the evolution of acidity, indicating that acidity levels in kefir are not static but fluctuate over time depending on storage conditions and treatment type.

These findings highlight the complexity of acidity development in kefir, as both the **type of flavoring** and **length of storage** influence acid production and stability. The impact of storage time underscores the progressive nature of lactic acid fermentation, which continues to adjust the acidity profile even after the initial fermentation period. The interaction between treatment and storage time, moreover, suggests that flavored kefir may exhibit different acidification dynamics than unflavored kefir, potentially enhancing the product's stability or extending its shelf life through sustained acidity.

In summary, adherence to Codex standards for lactic acid content is essential for quality assurance in kefir production. This study's findings provide insights into how the inclusion of natural additives, such as lemon extract, and prolonged storage may affect the acidity profile of kefir, contributing to improved understanding and management of acidification processes in fermented dairy products.

The **variation in acidity** over a 12-day refrigerated storage period at 4°C was examined in both control and lemon-flavored kefir, as illustrated in the scatter plot (Fig. 13). The **control kefir** exhibited a higher initial acidity of **8.8 g/L** compared to the flavored variant's **8.6 g/L**, and this natural kefir continued to show a consistent increase in acidity, reaching its peak of **9.6 g/L on Day 12**. Such progressive acidification aligns with previous research; for instance, Pourbaba et al. (2022) reported a comparable rise in the titratable acidity of cow's milk kefir from **0.80 g/100 g to 0.83 g/100 g** over a 14-day refrigerated period. This pattern of increasing acidity reflects ongoing lactic acid bacterial activity, which persists, albeit at a slower rate, during cold storage and continues to acidify the kefir over time.

In contrast, the **flavored kefir** exhibited fluctuating acidity levels throughout the storage period, with the lowest acidity observed at **8.4 g/L on Day 12**. This stability in acidity suggests that the inclusion of lemon extract may inhibit lactic acid bacterial activity, thus moderating acid production. The essential oils in lemon extract, particularly **lemon oil**, possess known antimicrobial properties, which may influence kefir's microbial ecosystem by selectively inhibiting certain bacterial populations while allowing yeast activity to persist. This microbial modulation is further supported by Wulansari et al. (2023), who observed significant decreases in acidity over a 14-day storage period in flavored kefir, potentially due to yeast metabolism reducing overall acid levels. Interestingly, these findings diverge from those of Putri, Setiani, and Warya (2020), who observed a significant decline in lactic acid content in natural kefir, with acidity decreasing from **2.94% to 2.46%** over a 16-day period at slightly higher cold storage temperatures (6–10°C).

Such conflicting results underscore the complexity of kefir's fermentation process and the influence of variables such as **microbial composition, milk source, and storage temperature** on acidity evolution. These discrepancies suggest that natural and lemon-flavored kefir's acidic profiles are highly sensitive to both external and internal factors, leading to variations in fermentation behavior and microbial dynamics during storage. According to Hachana et al. (2017) acidity in other fermented dairy products, such as yogurt, also evolves continuously during storage. Similarly, in kefir, the ongoing acidification in the control variant likely reflects the sustained activity of lactic acid bacteria in a balanced microbial environment. By contrast, in the lemon-flavored kefir, yeast populations may have influenced acidity reduction by metabolizing acids as storage time progressed, as suggested by Güzel-Seydim et al. (2000) who found that yeast growth in kefir could counterbalance acid production by LAB, leading to a slight pH increase over time.

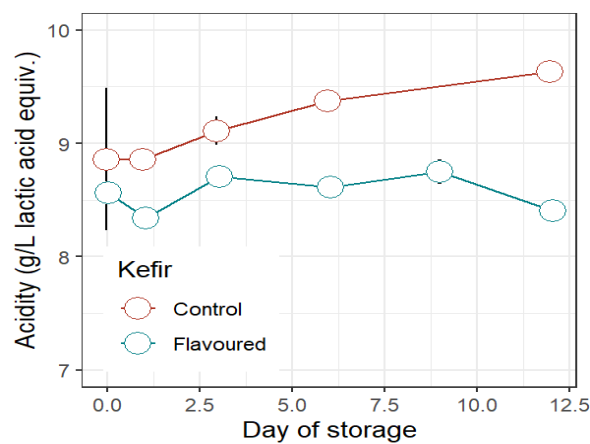


Figure 13: Evolution of kefir acidity during shelf life

Our findings indicate that while **natural kefir undergoes continuous acidification** during refrigerated storage, **lemon-flavored kefir exhibits relative stability in acidity** due to potential antimicrobial effects of lemon extract and yeast activity. This stabilization could be advantageous in prolonging kefir's sensory appeal by reducing excessive sourness, thereby enhancing product palatability over its shelf life. These insights suggest that natural flavor additives may serve as effective means to manage kefir's acidity profile, supporting both microbial stability and consumer satisfaction.

Table 14: Analyses of variance and least square means for the evolution of pH and ACIDITY in kefir

Types of kefir	Day	pH			ACIDITY				
		LS means	Lower CL	Upper CL	LS means	Lower CL	Upper CL		
Control	0	4.18	4.10	4.26	9.13	8.88	9.37		
	1	4.25	4.18	4.33	8.98	8.73	9.23		
	3	4.31	4.23	4.39	9.31	9.06	9.56		
	6	4.16	4.10	4.23	9.34	9.14	9.55		
	9	4.34	4.28	4.41	9.45	9.13	9.77		
	12	4.16	4.09	4.22	9.37	9.16	9.58		
Treatment	0	4.17	4.10	4.24	8.43	8.20	8.65		
	1	4.25	4.18	4.32	8.28	8.06	8.50		
	3	4.30	4.23	4.37	8.61	8.39	8.83		
	6	4.16	4.09	4.22	8.64	8.43	8.85		
	9	4.34	4.27	4.40	8.75	8.49	9.01		
	12	4.15	4.08	4.21	8.67	8.46	8.87		
Source of variation		num DF	den DF	F-value	p-value	num DF	den DF	F-value	p-value
Intercept		1	35	60175	<.0001	1	31	59813	<.0001
Treatment		1	35	0	0.9274	1	31	88	<.0001
Day		1	35	0	0.7618	1	31	11	0.0023
Treatment: Day		1	35	1	0.3572	1	31	14	0.0008

CL: Confidence Limit

LS means: Least Squares Means

num DF: Number of Degrees of Freedom

den DF: Denominator Degrees of Freedom

4.3. Syneresis

The **syneresis** of kefir, defined as the separation of liquid whey from the solid matrix, was monitored over a 12-day storage period to assess changes in structural stability. As shown in Table 15, **no significant effect of treatment** (control vs. lemon-flavored) on syneresis was observed ($p > 0.05$), while **storage period alone** significantly impacted syneresis values ($p < 0.05$). These results suggest that although lemon extract introduces additional acidity, it does not alone drive significant changes in syneresis when compared to the control kefir.

In Figure 14, both kefir types exhibit similar trends in syneresis evolution, starting with comparable initial values of **63-64% on Day 0** and increasing consistently over the storage period. By Day 12, syneresis reaches **86-89%**, with a slight but higher final value for the lemon-flavored kefir. This increase in syneresis during storage aligns with findings from Ozcan et al. (2018), who observed similar rises in syneresis for plain and fruit-flavored kefir samples, suggesting that syneresis naturally progresses over time as a result of protein structure changes and moisture migration.

The higher syneresis in the flavored kefir can likely be attributed to the **added acidity from lemon extract**, which may expedite the syneresis process. Increased acidity can impact protein interactions within the kefir matrix, leading to more pronounced phase separation between the whey and curd components. This effect was similarly noted by Dinkci et al. (2015), who reported a significant impact of storage on syneresis, particularly in the early days of storage, as acidification progresses and structural changes in protein networks become more apparent. The patterns observed in this study, with gradual increases in syneresis over time, underline the role of storage duration as a primary factor in kefir's structural stability. While the lemon extract's impact was modest, it suggests that **natural acidifiers** in flavored kefir may subtly influence syneresis without fundamentally altering the kefir's storage stability profile. These findings provide insights for kefir producers, particularly for formulating flavored products where acidity must be balanced to manage whey separation and maintain product texture over its shelf life.

In conclusion, this study demonstrates that **storage duration** is a key determinant of syneresis in kefir, with the addition of lemon extract slightly accelerating this process. These insights underscore the importance of monitoring acidity and structural changes to ensure optimal texture and stability, especially for flavored kefir varieties intended for extended shelf life.

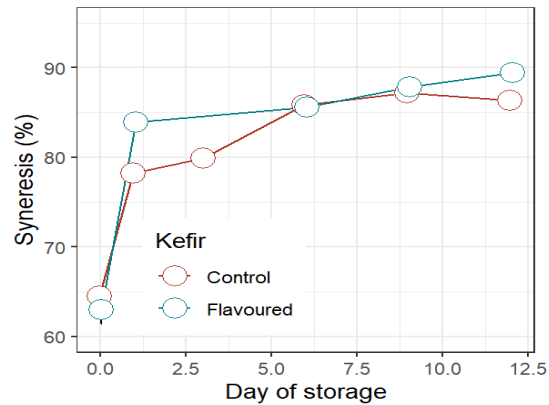


Figure 14: Evolution of kefir syneresis during shelf life

4.4. Proteolysis

The **OPA (o-phthaldialdehyde) spectrophotometric assay** was employed to assess **proteolytic activity** in kefir, providing a quantitative measure of α -amino groups released during the breakdown of milk proteins (Dinkci et al. 2015). Proteolysis is a critical process in kefir fermentation, as it influences texture, flavor, and nutrient release. Statistical analysis (Table 15) indicates that both **treatment type** (control vs. lemon-flavored kefir) and the **interaction between treatment and storage period** significantly impacted proteolysis levels ($p < 0.05$), suggesting that lemon extract and storage conditions together drive differences in protein breakdown. Figure 15 illustrates distinct proteolysis trends for the two kefir varieties, showing divergence from **Day 1** of storage. Notably, **control kefir** displayed a rapid and substantial increase in proteolysis, with values rising sharply from **0.15 to 0.49** within the first day—a threefold increase. This rapid proteolytic activity may be attributed to the added lemon extract, which could influence microbial dynamics or enzyme activity, accelerating protein degradation. By contrast, **flavored kefir** exhibited a moderate increase from **0.2 to 0.29**, followed by a stabilization around **0.2** for the remainder of the storage period, indicating minimal ongoing proteolysis.

The sustained and high proteolysis levels observed in control kefir, which reached **0.5 by Day 12**, reflect the influence of flavor additives on microbial and enzymatic processes. This significant divergence from the relatively stable proteolytic profile of the flavored kefir suggests that lemon extract may limit proteolytic activity by interacting with microbial populations or affecting the balance of enzymes involved in protein breakdown. Such interactions are in line with findings from Dinkci et al. (2015), who observed similar increases in proteolysis across different kefir samples, highlighting a consistent trend in the evolution of protein degradation during storage.

The concordance between our observations and previous research validates the trends noted in this study. **The stable proteolysis in flavoured kefir** can help maintain flavor, nutrient availability, and potential modifications in texture, underscoring the flavoring agent's role in altering kefir's biochemical profile during storage. Additionally, the interaction between flavoring, microbial composition, and storage duration may offer producers a means to control kefir's sensory attributes, achieving a tailored product profile that aligns with consumer preferences. This study confirms that lemon flavoring weakly influences proteolysis in kefir, resulting in stable protein degradation compared to control samples. These results contribute to a better understanding of the impact of natural flavor additives on the biochemical processes of kefir, offering insights for optimizing product formulation and enhancing consumer appeal.

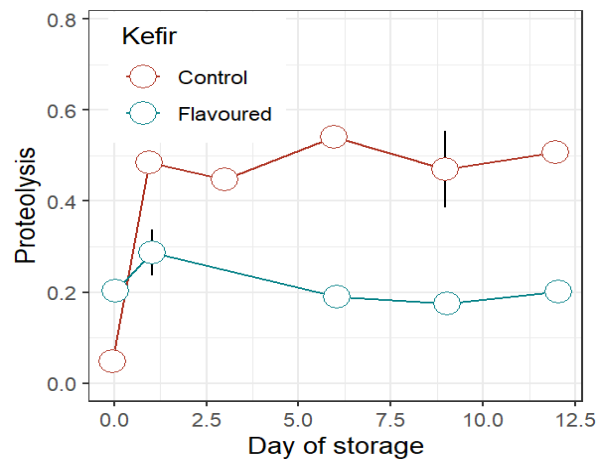


Figure 15: Evolution of kefir proteolysis during shelf life

5. Textural properties

A **comparative analysis** of scatterplots and ANOVA tables, conducted in RStudio, revealed significant differences in **texture parameters** between control and lemon extract-flavored kefir over a 12-day storage period. This analysis focused on key textural attributes, including **firmness, consistency, cohesiveness, and viscosity index**.

The scatterplots visually captured the distinct trends in these properties, while the ANOVA results confirmed statistically significant divergences, providing a comprehensive view of how flavoring and storage influence kefir's textural evolution.

Table 15: Analyses of variance and least square means for the evolution of SYNERESIS and PROTEOLYSIS in kefir

Types of kefir	Day	SYNERESIS			PROTEOLYSIS				
		LS means	Lower CL	Upper CL	LS means	Lower CL	Upper CL		
Control	0	63.0	61.0	65.0	0.2199	0.0851	0.355		
	1	80.3	78.4	82.3	0.4804	0.3456	0.615		
	3	79.9	77.3	82.4	0.4470	0.27440	0.620		
	6	85.0	83.0	86.9	0.4868	0.3406	0.633		
	9	86.8	84.8	88.8	0.4159	0.2811	0.551		
	12	87.1	85.2	89.1	0.4484	0.3136	0.583		
Treatment	0	64.5	62.6	66.5	0.0316	-0.1032	0.166		
	1	81.9	79.9	83.8	0.2921	0.1573	0.427		
	3	81.4	78.4	84.4	0.2586	0.0519	0.465		
	6	86.5	84.5	88.4	0.2984	0.1383	0.459		
	9	88.3	86.4	90.3	0.2276	0.0928	0.362		
	12	88.7	86.7	90.6	0.2601	0.1253	0.395		
Source of variation		num DF	den DF	F-value	p-value	num DF	den DF	F-value	p-value
Intercept		1	17	3874.8	<.0001	1	16	150.951	<.0001
Treatment		1	17	0.4	0.5435	1	16	14.034	0.0018
Day		1	17	27.0	0.0001	1	16	2.400	0.1409
Treatment: Day		1	17	0.0	0.8583	1	16	5.230	0.0362

CL: Confidence Limit

LS means: Least Squares Means

num DF: Number of Degrees of Freedom

den DF: Denominator Degrees of Freedom

The **graphical analysis** of texture data highlights the temporal variations in texture profiles between the two kefir types. For instance, viscosity index and consistency in the flavored kefir displayed unique patterns, with periodic fluctuations suggesting that lemon extract may interact with the kefir matrix, potentially enhancing or stabilizing certain textural qualities over time. In particular, flavored kefir showed greater resilience in cohesiveness and firmness during the latter half of the storage period, suggesting a possible **preservative effect** of lemon extract that may modulate microbial or enzymatic activity, thereby impacting texture stability.

Through detailed examination of the scatterplots, these differences in textural evolution were quantified, providing a robust foundation for evaluating the potential of **lemon extract as a natural texture modifier and preservative**. The ability of lemon extract to influence the textural properties of kefir could present an advantage in terms of shelf-life extension and consumer preference, as textural qualities are essential to kefir's sensory perception and palatability. By modifying texture, lemon extract may improve the **mouthfeel and viscosity** of kefir, resulting in a smoother, more consistent product that appeals to a broad range of consumers. The **rheological and textural properties** of kefir, including how it feels and flows in the mouth, significantly influence **consumer acceptance**. These qualities largely shape the sensory experience of kefir and are crucial determinants of customer satisfaction. Thus, texture and rheological characteristics are not merely physical properties but are integral to the **taste experience and overall consumer appeal**. This comprehensive approach to assessing kefir's textural evolution provides valuable insights for optimizing formulations with natural additives, aligning product characteristics with consumer preferences, and potentially enhancing marketability. The study's findings underscore the influence of lemon extract on **texture stability and sensory quality** in kefir, reinforcing the importance of textural properties in consumer perception and acceptance. These insights contribute to a growing body of knowledge on natural additives in dairy products, supporting their use in enhancing both the stability and sensory appeal of fermented beverages.

5.1. Firmness

Firmness is a critical textural attribute, referring to the resistance of a food product to deformation under applied force, and serves as an indicator of **quality and maturity** according to Codex Alimentarius standards (Mudgil, Barak, and Khatkar, 2017). In this study, firmness was evaluated as the force required to achieve a specified deformation in kefir, reflecting the internal structural integrity and bond resistance of the kefir matrix.

Statistical analysis (Table 16) indicates that **only the storage period** significantly influenced firmness ($p < 0.05$), suggesting that changes in firmness primarily resulted from storage

conditions rather than the presence of lemon flavoring. According to the dispersion curves (Fig. 16), the initial firmness of control and flavored kefir differed markedly, with control kefir displaying a **higher initial firmness of 11.9 g** compared to the flavored kefir's **9.4 g**. This difference highlights the potentially stabilizing effect of lemon extract on initial texture, as the flavored kefir began with a lower but more stable firmness level.

Throughout the storage period, **firmness values for both kefir types gradually converged**. The control kefir exhibited a sharp decline in firmness, reaching values close to or lower than the flavored variant, while the flavored kefir showed only minor decreases, stabilizing at **9.2 g by Day 1**. By the end of the 12-day period, both kefirs had converged to a **firmness value of approximately 9 g**, suggesting that the textural differences between control and flavored kefir diminished over time. This convergence may be attributed to ongoing fermentation processes or the impact of consistent storage conditions, which appear to equalize firmness by moderating the structure of both kefir types. The observed reduction in firmness aligns with findings by Ozcan et al. (2018), who reported a general decrease in firmness across both plain and flavored kefir samples during storage, with an average decline from **30.41 g to 19.67 g**. This trend underscores the influence of storage on textural softening in fermented dairy products, where prolonged fermentation and moisture redistribution can lead to a less rigid structure.

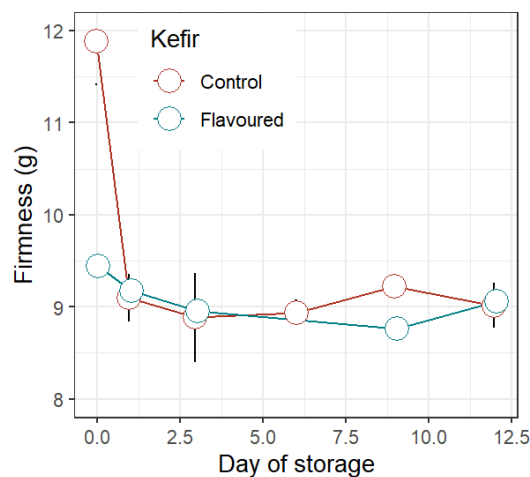


Figure 16: Evolution of kefir firmness during shelf life

In conclusion, while lemon flavoring did not independently affect the firmness parameter, storage duration significantly influenced the textural evolution of kefir, with both control and flavored varieties ultimately reaching similar firmness levels. These findings suggest that, despite initial textural variations, storage conditions exert a homogenizing effect on kefir

firmness over time. This insight may be valuable for producers aiming to achieve consistent texture across flavored and unflavored kefir products throughout their shelf life.

5.2. Consistency

Consistency is a key textural property of kefir, heavily influencing its **sensory appeal and consumer acceptance**. This attribute reflects the product's internal structure and viscosity, shaped by both **fermentation processes** and **storage conditions**. From the scatterplot (Fig. 17), we observe that at the beginning of storage (**Day 1**), the control kefir exhibited a higher consistency (**89 g·s**) compared to the lemon-flavored kefir (**70 g·s**), indicating a firmer and more cohesive structure in the initial stages for the control sample.

Over the 12-day storage period, the two kefir types followed **distinct trajectories** in terms of consistency. **Control kefir** showed a steady decline in consistency, culminating in a **minimum value of 55 g·s** on Day 12. This downward trend suggests ongoing structural breakdown, potentially due to **microbial and enzymatic activity** that progressively weakens the kefir matrix. In contrast, **flavored kefir** displayed an irregular pattern, with fluctuations in consistency throughout storage at 4°C. Interestingly, by Day 12, the flavored kefir had surpassed its initial consistency, reaching **72.5 g·s**. This unexpected increase implies that the presence of lemon extract may have influenced proteolytic or microbial activity, contributing to a distinct ripening process that differs from the control kefir.

This **divergence in consistency evolution** between control and flavored kefir highlights potential differences in **textural degradation or stabilization mechanisms** influenced by lemon flavoring. The findings from this study contrast with those of Ozcan et al. (2018), who reported a decrease in consistency for both plain and flavored kefir samples by the end of storage. The presence of lemon extract in our study's flavored kefir may have interacted with microbial populations or slowed down matrix degradation, yielding a consistency that remains more stable over time.

Statistical analysis (Table 16) further substantiates these observations, revealing that **storage duration, treatment type, and their interaction** all had a significant impact on kefir consistency ($p < 0.05$). This result underscores the complexity of kefir's textural evolution, as both storage and flavor additives can interact to shape the product's physical characteristics. The significant interaction between treatment and storage duration suggests that flavoring agents, such as lemon extract, could potentially act as stabilizers under certain conditions, impacting proteolysis, microbial balance, or moisture migration within the product.

These findings underscore the importance of carefully **considering both storage conditions and processing methods** when evaluating kefir's textural properties. By understanding how

different factors contribute to consistency, producers can better predict and control textural changes over time, enhancing product quality and consumer satisfaction. The potential stabilizing effect of lemon flavoring offers a valuable insight into formulation strategies for flavored kefir varieties, supporting efforts to achieve consistent texture and extended shelf life. In summary, this study demonstrates that while **control kefir undergoes steady softening**, flavored kefir shows a more **resilient texture**, potentially influenced by the lemon extract. This insight into consistency evolution in kefir emphasizes the role of natural additives in modifying and potentially enhancing textural stability, which is crucial for product appeal and marketability.

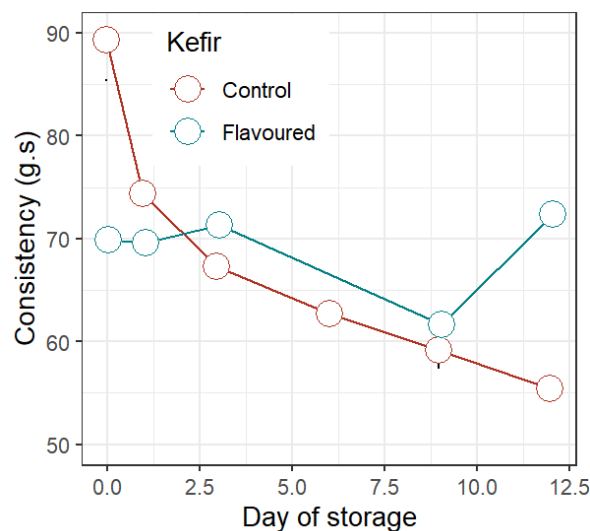


Figure 17: Evolution of kefir consistency during shelf life

5.3. Cohesiveness

Cohesiveness refers to the **internal bonding forces** that uphold a product's structural integrity, reflecting its capacity to retain shape under stress and the strength of its molecular interactions (Domagała et al., 2006). This textural property is essential for assessing the resilience and mouthfeel of kefir, as well as for understanding how processing and storage conditions might impact its overall texture. Based on the **ANOVA results** (Table 17), none of the factors studied—**treatment type, storage period, or their interaction**—had a statistically significant effect on cohesiveness ($p > 0.05$). This finding suggests that cohesiveness in kefir is relatively stable over the storage period, showing resilience to variations in both flavoring and storage time.

Table 16: Analyses of variance and least square means for the evolution of FIRMNESS and CONSISTENCY in kefir

Types of kefir	Day	FIRMNESS			CONSISTENCY				
		LS means	Lower CL	Upper CL	LS means	Lower CL	Upper CL		
Control	0	10.58	10.07	11.09	75.4	69.3	81.5		
	1	9.47	8.97	9.98	70.2	64.1	76.3		
	3	9.26	8.75	9.77	69.0	62.9	75.1		
	6	8.94	8.40	9.48	62.7	56.2	69.2		
	9	9.23	8.81	9.66	59.6	54.5	64.7		
	12	9.28	8.85	9.71	63.1	58.0	68.2		
Treatment	0	10.09	9.63	10.55	76.9	71.4	82.4		
	1	8.99	8.53	9.45	71.7	66.2	77.2		
	3	8.77	8.31	9.23	70.5	65.0	76.0		
	6	8.45	7.79	9.11	64.2	56.3	72.1		
	9	8.75	8.32	9.18	61.1	56.0	66.2		
	12	8.79	8.37	9.22	64.6	59.5	69.7		
Source of variation		num DF	den DF	F-value	p-value	num DF	den DF	F-value	p-value
Intercept		1	31	8080.9	<.0001	1	31	7819.2	<.0001
Treatment		1	31	1.8	0.11866	1	31	6.7	0.0148
Day		1	31	7.7	0.0092	1	31	36.0	<.0001
Treatment: Day		1	31	2.9	0.0973	1	31	35.4	<.0001

CL: Confidence Limit

LS means: Least Squares Means

num DF: Number of Degrees of Freedom

den DF: Denominator Degrees of Freedom

The lack of statistically significant effects may imply that the influences of treatment and storage duration are minimal or that any changes are too subtle to detect with the current sample size and study design. However, it is also plausible that **external factors not accounted for in this study** could play a larger role in determining kefir's cohesiveness. Despite the absence of significant effects, the **cohesiveness curves** (Fig. 18) reveal an interesting trend over time. Initially, the control kefir exhibited a **significantly higher cohesiveness** (7.8 g) than the flavored kefir (2.9 g). On Day 1, however, a **notable reversal** occurred: the control kefir's cohesiveness dropped sharply to **4.5 g**, while the flavored kefir's cohesiveness increased to **5.6 g**, surpassing the control sample. From this point, both kefir varieties followed a similar stability trend, with cohesiveness values converging and stabilizing at around **5.5–5.6 g** by the third day of storage. This stability continued until the end of the storage period (Day 12), suggesting that **cold storage promotes textural convergence** in kefir, leading both variants toward similar cohesiveness values over time. These observations imply that the addition of lemon flavoring does not significantly alter the cohesiveness of kefir in the long term. Although initial differences were evident, storage conditions at **4°C** appear to have a **homogenizing effect** on cohesiveness, likely due to structural adjustments in the protein and microbial matrix during cold storage. This finding aligns with the study by Ozcan et al. (2018), which reported higher cohesiveness in fruit-flavored kefir samples by the end of storage, indicating that flavored kefir samples can retain cohesiveness similar to or higher than plain samples under specific conditions. In conclusion, while **lemon flavoring initially influenced cohesiveness**, both flavored and control kefir samples stabilized to comparable cohesiveness levels over the storage period. This convergence indicates that **storage duration may exert a greater impact on cohesiveness than flavoring** in kefir, with cold storage contributing to a uniform texture across variants. These results are valuable for producers seeking to maintain consistent texture in both plain and flavored kefir products throughout shelf life, enhancing both quality control and consumer satisfaction.

5.4. Index of viscosity

Viscosity is a primary rheological parameter in fermented milk products, significantly impacting both **product quality and consumer acceptance** (Ozcan, Yilmaz-Ersan, Akpinar Bayazit, et al., 2018). In kefir, viscosity reflects the **thickness and resistance to flow**, characteristics that are influenced by microbial activity, structural integrity, and storage conditions. Analysis of variance (ANOVA) results (Table 17) indicate that **storage time** and its **interaction with treatment type** had a statistically significant impact on kefir's viscosity ($p < 0.05$), suggesting that viscosity fluctuates over time and that its evolution depends on the

specific treatment applied to each kefir variant. According to the scatterplots (Fig. 19), the initial viscosity values differed notably between control and lemon-flavored kefir. **Control kefir** displayed an initial viscosity of **-0.95 g·s**, while **flavored kefir** had a lower initial viscosity of **-1.57 g·s**. This indicates that the control kefir was initially thicker, possibly due to differences in **microbial composition or matrix stability** between the two treatments.

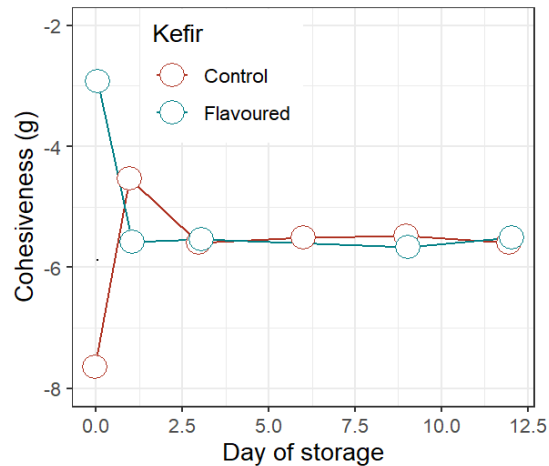


Figure 18: Evolution of kefir cohesiveness during shelf life

Over the 12-day storage period at 4°C , viscosity trends diverged between the two kefir types. **Control kefir** showed fluctuating viscosity levels, but with an overall downward trend, ultimately reaching **-0.97 g·s** on Day 12. This decrease suggests a gradual weakening of the kefir structure, likely due to **protein degradation or enzymatic activity** over prolonged storage. Such changes may reflect alterations in the rheological properties of control kefir, impacting its mouthfeel and consumer perception. In contrast, the **flavored kefir** exhibited fluctuations in viscosity as well, but with a distinct outcome: by Day 12, its viscosity increased beyond the initial measurement, reaching **-0.89 g·s**. This upward trend could indicate that **chemical interactions between lemon flavoring compounds and kefir's structural components** contribute to viscosity stabilization or even enhancement over time. These changes may also stem from **modifications in the microbial ecosystem** influenced by lemon extract, which could affect the structural properties of the kefir matrix. These observations align partially with findings from Dinkci et al. (2015), who noted that samples with varying oat milk concentrations reached their peak viscosity at the beginning of storage, while viscosity decreased with storage. Additionally, Tratnik et al. (2006) found that **goat's milk kefir products had lower viscosity** than cow's milk kefir, indicating that milk type plays a role in viscosity behavior. Meanwhile, Putri, Setiani, and Warya (2020) demonstrated that viscosity increases in goat milk kefir were **time-dependent**, emphasizing the combined effects of storage

duration and temperature on kefir's rheological properties. According to their findings, kefir can be stored up to 24 days without additives, with an optimal shelf life of 4–12 days, aligning with the time frame of our study. The results of this study reveal that **lemon flavoring not only affects the initial viscosity** of kefir but also influences its evolution throughout storage. The decrease in viscosity observed in control kefir is likely linked to **protein degradation** or structural weakening due to extended storage. In flavored kefir, however, **increased viscosity** may result from the presence of lemon extract, potentially enhancing stability or modifying microbial and chemical interactions within the kefir matrix. These findings suggest a need for further exploration into the mechanisms underpinning viscosity variations, such as chemical interactions and microbial activity shifts influenced by flavoring. In conclusion, the study illustrates that **flavoring can significantly alter the viscosity profile** of kefir over time, offering potential avenues for tailored viscosity management in flavored kefir products. Understanding these factors is essential for developing kefir with controlled rheological properties, ensuring optimal quality and consumer satisfaction throughout the product's shelf life.

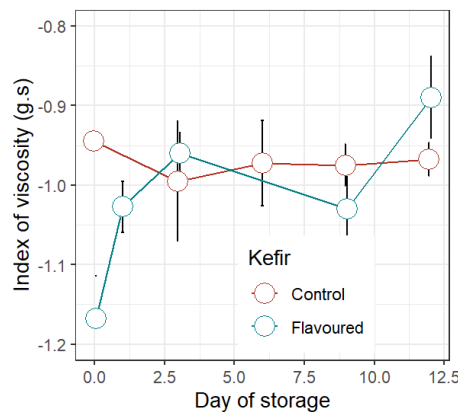


Figure 19: Evolution of kefir viscosity index during shelf life

6. Microbiological properties

The **microbiological evolution** of kefir is a crucial indicator of product safety, shelf stability, and quality, with microbial profiles shaping both the sensory properties and health benefits of fermented dairy products. In this study, a comparative analysis of **control and lemon extract flavored kefir** was conducted over a **13-day storage period**, with significant differences observed in their microbiological profiles. Utilizing **ANOVA and scatter plots** generated in RStudio, this analysis provided a detailed look at changes in **lactic acid bacteria (LAB), mesophiles, yeasts, and molds**, allowing for the quantification of these variations over time.

Table 17: Analyses of variance and least square means for the evolution of COHESIVENESS and VISOCITY INDEX in kefir

Types of kefir	Day	COHESIVENESS			VISCOSITY INDEX				
		LS means	Lower CL	Upper CL	LS means	Lower CL	Upper CL		
Control	0	-4.90	-6.89	-2.91	-1.073	-1.164	-0.982		
	1	-5.63	-7.62	-3.64	-0.997	-1.116	0.878		
	3	-5.95	-7.94	-3.69	-0.951	-1.042	-.860		
	6	-5.51	-7.62	-3.40	-0.973	-1.066	-0.879		
	9	-5.87	-7.54	-4.20	-0.987	-1.063	-0.911		
	12	-5.85	-7.52	-4.18	-0.913	-0.989	-0.838		
Treatment	0	-4.30	-6.09	-2.51	-1.104	-1.184	-1.023		
	1	-5.03	-6.82	-3.23	-1.028	-1.121	0.934		
	3	-5.35	-7.14	-3.55	-0.982	-1.062	-0.901		
	6	-.491	-7.50	-2.33	-1.003	-1.122	-0.884		
	9	-5.27	-6.94	-3.60	-1.018	-1.094	-0.942		
	12	-5.25	-6.92	-3.58	-0.944	-1.020	-0.868		
Source of variation		num DF	den DF	F-value	p-value	num DF	den DF	F-value	p-value
Intercept		1	31	244.960	<.0001	1	29	4508.1	<.0001
Treatment		1	31	0.998	0.3255	1	29	2.1	0.1534
Day		1	31	0.451	0.5067	1	29	6.5	0.0166
Treatment: Day		1	31	1.468	0.2348	1	29	4.1	0.0517

CL: Confidence Limit

LS means: Least Squares Means

num DF: Number of Degrees of Freedom

den DF: Denominator Degrees of Freedom

The graphical representations highlight notable differences in microbial populations between the two kefir types. For instance, **lactic acid bacteria** and **mesophiles** exhibited distinct trends in each sample, suggesting that lemon extract potentially influenced microbial growth dynamics. In particular, the lemon-flavored kefir displayed a unique microbiological pattern, with **inhibition of certain microbial populations** such as yeasts and molds compared to the control. This effect is likely attributable to the **antimicrobial properties of lemon extract**, which contains natural compounds known for their preservative effects, potentially curbing microbial growth and modifying the kefir's microbial ecosystem. An **in-depth analysis** of these data provided a robust basis for evaluating the **efficacy of lemon extract as a natural preservative**. By quantifying changes in microbial populations, the study demonstrated how lemon flavoring could enhance **microbial stability** in kefir, potentially extending its shelf life by limiting the growth of spoilage microorganisms. This stabilization effect is crucial for manufacturers aiming to produce flavored kefir with consistent quality and extended storage potential. Furthermore, these findings suggest that flavoring kefir with natural additives like lemon extract may allow producers to maintain lower yeast and mold levels, which can otherwise lead to undesirable changes in texture and taste over time. This study's approach to **evaluating flavoring effects on microbiological stability** contributes to a nuanced understanding of the **interactions between natural preservatives and microbial behavior** in kefir. The findings indicate that flavoring agents not only influence sensory attributes but may also serve as functional ingredients that support product quality by enhancing microbial stability. This insight could guide further research into flavor-based preservation strategies for kefir, allowing producers to develop more stable and appealing flavored kefir products. In summary, this comparative analysis of kefir's microbiological properties demonstrates that **lemon extract can significantly impact the microbial stability** of kefir, reducing the presence of spoilage organisms while preserving beneficial microbial populations. This outcome highlights the dual role of lemon extract as a **flavor enhancer and potential preservative**, underscoring the value of natural additives in optimizing both the sensory and microbiological qualities of kefir.

6.1. Lactic acid bacteria

Lactic acid bacteria (LAB) are essential to kefir's fermentation process, contributing to its **flavor, texture, preservation, and health benefits**. The microbial diversity of kefir grains is extensive, with LAB as a dominant group. Common LAB species in kefir include *Lactobacillus paracasei*, *Lactobacillus acidophilus*, *Lactobacillus delbrueckii*, *Lactobacillus plantarum*, *Lactobacillus kefirianofaciens*, and *Lactobacillus kefir* (Prado et al. 2015). These bacteria

produce **lactic acid**, a crucial component for maintaining the product's quality by influencing sensory and structural attributes.

Statistical analysis (Table 18) indicates that **storage duration** and its **interaction with treatment type** significantly affect LAB concentrations ($p < 0.05$). This finding implies that lemon extract flavoring and storage time together influence the microbial stability of kefir. The scatterplots (Fig. 20) reveal that both kefir types began with **high initial LAB concentrations**, with the control at **9 log CFU/mL** and the flavored kefir slightly higher at **9.3 log CFU/mL**. However, over the 13-day storage period, LAB levels gradually declined, reaching **7.7 log CFU/mL** in the control and **7.4 log CFU/mL** in the flavored kefir on the final day.

The observed reduction in LAB concentrations over time is typical in refrigerated fermented products, where **low temperatures (4°C)** slow down microbial activity, leading to a gradual decrease in viable LAB counts. Similar results were reported by Irigoyen et al. (2005), who found a decline in LAB populations in kefir samples with varying grain/milk ratios (1% and 5%) between 7 and 14 days of storage. This decline in LAB viability during refrigeration highlights the challenges of maintaining active microbial populations in fermented dairy products over extended storage periods. However, some studies have shown contrasting results. For instance, Guzel-Seydim et al. (2005) observed that LAB in Turkish kefir were remarkably resilient during refrigerated storage, with microbial counts not only remaining stable but even exhibiting growth after 21 days. This resilience could be attributed to **differences in microbial composition** or environmental factors unique to the Turkish kefir sample, which may influence LAB adaptation to cold storage. Additionally, Ponomarova et al. (2017) noted that **yeast presence can favorably impact LAB** by providing growth stimulants and metabolizing some of the lactic acid produced, thus creating a supportive environment for LAB longevity. The results of this study indicate that **lemon-flavored kefir began with a slightly higher LAB concentration**, which could be attributed to initial differences in microbial load or the effect of flavoring on microbial growth in the early stages. However, both control and flavored kefir samples showed similar declining trends, suggesting that **cold storage exerts a stronger influence on LAB stability** than flavoring. These findings provide insights into the dynamics of LAB in kefir, highlighting the need for optimal storage conditions to maintain microbial quality and ensure health benefits. In conclusion, this study reveals that while **lemon flavoring initially increased LAB levels**, the impact of refrigerated storage at 4°C led to a general decline in LAB concentrations for both kefir types. The stabilizing effect of cold storage on LAB populations in kefir aligns with prior findings yet highlights potential differences in LAB resilience across kefir varieties. These results are significant for producers aiming

to preserve LAB viability in flavored kefir, contributing to product quality, shelf life, and probiotic benefits.

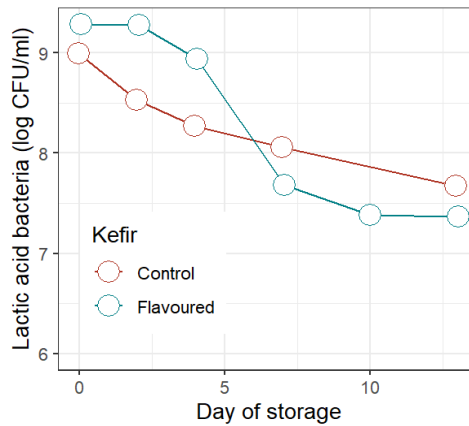


Figure 20: Evolution of kefir lactic acid bacteria during shelf life

6.2. Mesophiles

Kefir's complex microbial ecosystem includes a variety of **mesophilic bacteria**, which contribute to its unique sensory and probiotic qualities. Among these bacteria, species from the genera *Lactobacillus*, *Lactococcus*, *Leuconostoc*, and *Streptococcus* are particularly prevalent, playing an essential role in the **fermentation process** and stability of the product. According to the analysis of variance (Table 18), **storage duration and its interaction with treatment type** had a statistically significant impact ($p < 0.05$) on mesophilic bacterial populations, indicating that these factors jointly influence mesophilic bacterial stability in kefir.

The significant interaction between **storage time and treatment** highlights that the effect of storage duration on mesophilic bacteria is not uniform across both kefir types. This interaction implies that lemon flavoring impacts the rate at which mesophilic bacteria decline over the storage period, providing essential insights for optimizing **product quality and shelf life** to preserve kefir's microbial benefits. The **scatterplots** (Fig. 21) show that at the beginning of storage, both kefir types had high mesophilic concentrations, with the control kefir showing an initial count of **9.1 log CFU/mL** and the flavored kefir slightly higher at **9.3 log CFU/mL**.

This high initial mesophilic load reflects the substantial microbial activity immediately following fermentation. However, as storage at **4°C** progressed, mesophilic populations in both kefir types began a gradual decline. The flavored kefir experienced a more pronounced reduction in mesophilic bacteria compared to the control kefir, reaching a final count of **7.2 log CFU/mL** by Day 13, whereas the control kefir had a slightly higher concentration of **7.6 log CFU/mL**. The **faster decline in mesophiles** in the flavored kefir may be attributed to factors related to lemon extract, such as the **interaction with aromatic compounds** that

can inhibit certain bacterial strains, or a **pH modification** that creates a less favorable environment for bacterial survival. These effects are consistent with findings from Dinkci et al. (2015), who observed a significant decrease in *Lactococcus spp.* populations over 21 days in cow's milk kefir stored under refrigeration. The decline in mesophilic populations during cold storage may also be driven by **progressive environmental acidification** due to lactic acid accumulation, microbial competition among kefir's various microorganisms, and gradual nutrient depletion.

Cold storage slows down metabolic activity in mesophilic bacteria but does not entirely halt it, allowing **slow fermentation** to continue even at low temperatures (Jebel, 2022). This decelerated fermentation contributes to gradual changes in mesophilic populations, as mesophiles adapt to the refrigerated environment with limited growth and survival under low-nutrient, high-acid conditions. In summary, this study reveals that **lemon-flavored kefir exhibits a faster decline in mesophilic bacteria** than control kefir during refrigerated storage, likely due to the added flavoring compounds and pH changes introduced by lemon extract. This finding suggests that lemon extract may act as a **mild inhibitory agent** on mesophilic populations, potentially extending shelf life by reducing microbial activity while still preserving beneficial probiotic effects. Such insights are valuable for manufacturers seeking to balance flavoring with microbial stability, supporting the development of kefir products with **enhanced shelf life** and consistent quality.

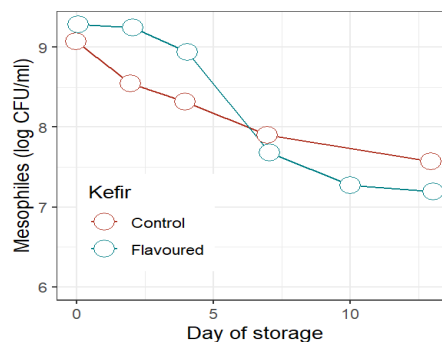


Figure 21: Evolution of kefir mesophiles during shelf life

6.3. Yeasts

Species like *Saccharomyces cerevisiae* are dominant in kefir and can ferment lactose and other sugars (Leite et al. 2012), thriving under conditions of **low pH, water activity (Aw), and temperature** (Rosset et al. 2002). Based on the ANOVA results (Table 19), **storage time and treatment type** significantly influenced ($p < 0.05$) yeast concentration in kefir, highlighting the combined impact of flavoring and storage duration on yeast behavior.

Table 18: Analyses of variance and least square means for the evolution of MESOPHILES and LACTIC ACID BACTERIA in kefir

Types of kefir	Day	MESOPHILES			LACTIC ACID BACTERIA				
		LS means	Lower CL	Upper CL	LS means	Lower CL	Upper CL		
Control	0	9.14	8.93	9.36	9.11	8.88	9.35		
	2	8.94	8.73	9.16	8.95	8.72	9.19		
	4	8.66	8.45	8.87	8.64	8.41	8.88		
	7	7.74	7.56	7.92	7.81	7.62	8.01		
	10	7.17	6.89	7.44	7.27	6.96	7.57		
	13	7.33	7.15	7.51	7.46	7.27	7.66		
Treatment	0	9.25	9.06	9.44	9.23	9.02	9.44		
	2	9.05	8.86	9.24	9.07	8.86	9.28		
	4	8.77	8.57	8.96	8.76	8.54	8.97		
	7	7.84	7.67	8.02	7.93	7.73	8.12		
	10	7.27	7.05	7.50	7.38	7.13	7.63		
	13	7.44	7.26	7.61	7.58	7.38	7.77		
Source of variation		num DF	den DF	F value	p-value	num DF	den DF	F value	p-value
Intercept		1	31	43132	<.0001	1	31	40852	<.0001
Treatment		1	31	3	0.091	1	31	3	0.1051
Day		1	31	334	<.0001	1	31	263	<.0001
Treatment: Day		1	31	25	<.0001	1	31	24	<.0001

CL: Confidence Limit

LS means: Least Squares Means

num DF: Number of Degrees of Freedom

den DF: Denominator Degrees of Freedom

As illustrated in the yeast dispersion curves (Fig. 22), the **initial concentration of yeast** differed between the two kefir types, with the control kefir starting at a **higher concentration** than the flavored variant. During the first 10 days of storage, yeast populations in both types exhibited a marked decline, likely due to environmental conditions at 4°C, which slow yeast metabolism and growth. However, from **Day 10 onwards**, yeast populations began to **increase progressively in the control** and grew **exponentially in the flavored kefir**. This upward trend continued until **Day 13**, with both kefir types reaching similar yeast concentrations by the end of the storage period, despite different starting points.

This observed yeast growth over time aligns with findings from Ozcan et al. (2018) who noted higher yeast counts in flavored kefir than in control samples during storage. The exponential growth observed in flavored kefir toward the end of the study may be attributed to **interactions between flavoring compounds and yeast cells**. For example, lemon extract could initially exert an inhibitory effect, delaying yeast growth, but as storage progresses, this effect may wane, allowing yeast to thrive under favorable conditions created by available lactose and stable pH. The study by Dinkci et al. (2015) supports this observation, showing that increased lactose availability positively influences yeast viability in kefir, likely by providing a continuous substrate for fermentation. Interestingly, studies on yeast stability during storage reveal diverse trends. For instance, **Solanki, Ghosh, and Kumawat (2023)** found that flavored kefir maintained sensory appeal longer than control kefir, which was rejected on Day 12 due to strong yeast and pungent flavors. This suggests that flavoring could modulate yeast activity to achieve prolonged freshness. Conversely, **Irigoyen et al. (2005)** observed no significant yeast count changes over 30 days in kefir stored at 5°C, while **O'Brien (2012)** reported a significant decline in yeast populations in traditionally prepared kefir over 30 days. These variations reflect the influence of storage conditions, microbial compositions, and flavoring agents on yeast dynamics in kefir. The trend toward **equalized yeast populations by the end of the storage period** suggests that both kefir variants reach a point of microbial equilibrium under refrigerated conditions, irrespective of initial treatment.

The lemon-flavored kefir's ability to achieve similar yeast counts by Day 13, despite a lower starting concentration, highlights the potential of lemon extract to **initially regulate yeast growth**, offering producers a method to manage yeast levels in flavored kefir without compromising microbial activity over time. In conclusion, this study illustrates that **flavoring agents influence initial yeast concentration and growth patterns** in kefir, with lemon extract delaying early yeast activity while ultimately allowing populations to stabilize. These findings

underscore the need to understand flavoring's role in microbial dynamics, as it may offer a valuable tool for **extending kefir shelf life and maintaining sensory appeal** by moderating yeast activity. The insights gained here contribute to the development of flavor-enhanced kefir products with optimized microbial and sensory profiles.

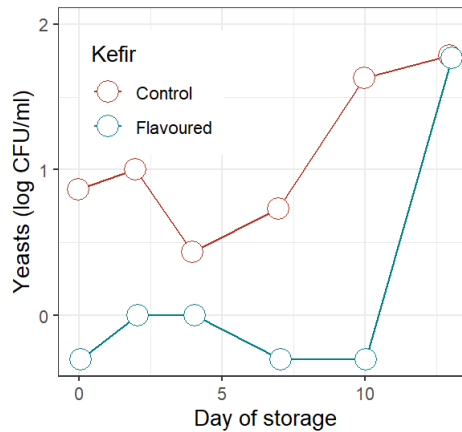


Figure 22: Evolution of kefir yeasts during shelf life

6.4.Molds

Molds are multicellular, filamentous fungi commonly associated with **spoilage in dairy products** due to their ability to grow under challenging conditions such as **low temperatures, low water activity, and low pH** (Shi and Maktabdar, 2022). Many molds responsible for dairy spoilage belong to the *Penicillium* and *Mucor* genera, among others, and can cause significant quality deterioration in fermented products (Garnier, Valence, and Mounier, 2017). However, in this study, **mold concentrations in kefir were unaffected by storage time or treatment type** ($p > 0.05$), suggesting that neither cold storage nor lemon flavoring significantly influenced mold growth in kefir (Table 19). According to the scatterplots (Fig. 23), initial mold presence was minimal, with molds barely detected in the lemon-flavored kefir and absent in the control sample. Both kefir types followed distinct trajectories over the storage period, with **control kefir reaching a peak concentration of 1 log CFU/mL by Day 7**, while **flavored kefir showed a slower increase, peaking at 0.6 log CFU/mL on Day 10**. After these peaks, mold concentrations in both variants declined, reaching **0 log CFU/mL by Day 13**. This decline may be attributed to the **antifungal activity of lactic acid bacteria (LAB)**, which naturally inhibit mold growth in fermented dairy products. The ability of LAB to act as a protective culture in kefir is well-documented, providing a **natural bioprotective effect** that can suppress or slow the growth of spoilage molds through various mechanisms. LAB produce **organic acids, bacteriocins, and other antimicrobial compounds** that create an unfavorable

environment for mold growth, particularly under cold storage conditions. This effect aligns with the findings of Shi and Knøchel (2021), who noted that LAB interactions with spoilage fungi are complex, with antifungal activity varying based on microbial composition and environmental conditions. This complexity underscores the role of LAB as an effective, albeit selective, biocontrol strategy in fermented products like kefir. The slower initial growth and lower peak concentration of molds in flavored kefir may also be influenced by **lemon extract's antimicrobial properties**, which could further restrict mold growth. Flavoring agents, such as essential oils or extracts, often contain compounds with antifungal activity, potentially enhancing kefir's resistance to mold proliferation. Although this effect was not statistically significant, it suggests that **lemon flavoring may contribute to a mildly inhibitory environment for mold**, supplementing the antifungal effects of LAB without compromising the microbial integrity of the product.

In conclusion, the findings indicate that **neither storage nor flavoring significantly altered mold populations** in kefir, with both control and flavored kefir ultimately converging to undetectable mold levels by the end of the storage period. The natural antifungal properties of LAB, combined with possible mild inhibitory effects of lemon flavoring, contribute to a **protective environment against mold growth**. These insights highlight the effectiveness of LAB as a natural defense against spoilage molds, emphasizing the potential for bioprotective cultures in maintaining the microbial quality and safety of fermented dairy products like kefir.

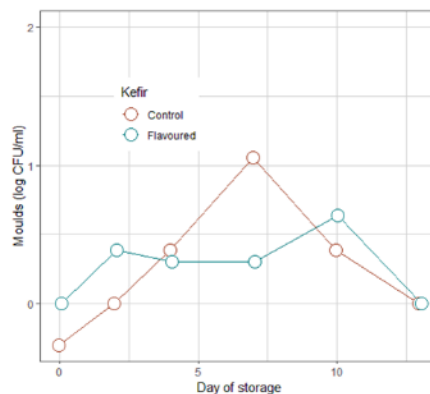


Figure 23: Evolution of kefir molds during shelf life

7. Sensory properties

The global popularity of kefir continues to grow, largely due to its **distinct sensory properties** and **health benefits**. However, goat's milk kefir presents a unique challenge in consumer acceptability, as it retains a characteristic "goaty" flavor that some individuals find unappealing.

Table 19: Analyses of variance and least square means for the evolution of YEASTS and MOLDS in kefir

Types of kefir	Day	YEASTS			Molds				
		LS means	Lower CL	Upper CL	LS means	Lower CL	Upper CL		
Control	0	0.7174	0.3975	1.0374	-0.0863	-0.2845	0.1118		
	2	0.9623	0.6424	1.2823	0.2734	0.0753	0.4715		
	4	0.7736	0.4536	1.0935	0.3444	0.1463	0.5425		
	7	0.6886	0.4189	0.9583	0.6874	0.5204	0.8544		
	10	1.1370	0.8673	1.4067	0.5222	0.3552	0.6892		
	13	2.2489	1.9792	2.5186	0.0105	-0.1565	0.1775		
Treatment	0	-0.2261	-0.5197	0.0675	-0.1074	-0.2892	0.0745		
	2	0.0188	-0.2748	0.3124	0.2524	0.0706	0.4342		
	4	-0.1699	-0.4636	0.1237	0.3234	0.1416	0.5052		
	7	-0.2549	-0.5246	0.0148	0.6664	0.4994	0.8334		
	10	0.1935	-0.0762	0.4632	0.5012	0.3342	0.6682		
	13	1.3054	1.0357	1.5751	-0.0105	-0.1775	0.1565		
Source of variation		num DF	den DF	F-value	p-value	num DF	den DF	F-value	p-value
Intercept		1	35	57.607	<.0001	1	35	30.0520	<.0001
Treatment		1	35	43.676	<.0001	1	35	0.2945	0.5908
Day		1	35	31.618	<.0001	1	35	0.5202	0.4755
Treatment: Day		1	35	0.183	0.6718	1	35	0.0976	0.7566

CL: Confidence Limit

LS means: Least Squares Means

num DF: Number of Degrees of Freedom

den DF: Denominator Degrees of Freedom

Transforming goat's milk into fermented products, such as kefir, with added flavors or supplements, can enhance both its sensory profile and nutritional value (Tratnik et al. 2006). In this study, a **sensory evaluation** of control and lemon-flavored kefir was conducted to gain insights into consumer preferences and optimize kefir's sensory attributes.

Panelists from IPB/CIMO assessed both kefir types based on **appearance, odor, taste, sourness, smoothness, and overall acceptability**. The evaluation results, analyzed by ANOVA, revealed statistically significant differences ($p < 0.05$) between the two kefir types in three primary sensory attributes: **odor ($p = 0.0041$), appearance ($p = 0.01$), and taste ($p = 0.047$)** (Table 20). A radar or spider-web diagram (Fig. 24) visually represented the sensory profiles, illustrating the intensity of these attributes for each kefir type on a unified graph.

Key Findings:

- **Odor:** Panelists rated the odor of flavored kefir significantly higher than the control. This aligns with the idea that lemon extract may mask or soften the strong, "goaty" aroma typical of goat's milk, making it more palatable to a broader range of consumers. The significance of this finding suggests that flavoring can be a valuable tool in enhancing the sensory appeal of goat's milk kefir.
- **Appearance:** Flavored kefir also scored higher in appearance, possibly due to slight visual differences introduced by the addition of lemon extract. This sensory aspect could play an important role in consumer perception, as appearance often sets expectations for flavor and quality.
- **Taste:** The flavored kefir received higher taste scores, likely due to the refreshing, citrus note from lemon extract, which balances and complements the sourness of kefir, enhancing overall palatability. This finding supports the use of natural flavor additives to broaden kefir's flavor profile and appeal.

Interestingly, the **overall acceptability** rating was close to significance ($p = 0.062$), suggesting a mild but notable preference for the flavored kefir. Although **sourness and smoothness** ratings were statistically similar between the two kefir types, the enhanced odor, appearance, and taste made the flavored variant the preferred choice among panelists. This preference aligns with findings by Irigoyen et al. (2005) who noted that kefir samples maintained high acceptability in the initial days of storage. The panelists' preference for attributes like milky taste, a pleasant odor, and balanced viscosity resonates with earlier research by Muir, Tamime, and Wszolek (1999) who identified these factors as essential for consumer satisfaction in fermented milk products, including kefir.

In conclusion, this sensory evaluation demonstrates that **lemon-flavored kefir is well-received** due to its enhanced sensory profile, especially in terms of odor, appearance, and taste. These results underscore the potential of flavoring to improve the appeal of goat's milk kefir, making it more acceptable to consumers who may otherwise find the natural aroma and taste too intense. This study thus provides a foundation for **using natural flavor additives to boost consumer preference and broaden kefir's market potential**, with applications in both product development and sensory optimization.

Table 20: Descriptive statistics and analysis of variances of the sensory attributes of kefir

Attribute	Control kefir		Flavored kefir		ANOVA Pr (>F)
	Mean	SD	Mean	SD	
Appearance	7.00	1.171	6.08	2.060	0.01*
Odor	5.22	1.805	6.48	2.246	0.0041**
Taste	4.23	2.034	5.10	2.059	0.047*
Sourness	5.09	1.87	5.45	1.698	0.34
Smoothness	5.40	2.012	5.56	2.050	0.71
Acceptance	4.76	2.17	5.58	1.972	0.062 .

***: p-value between 0 and 0.001 (highly significant)

**': p-value between 0.001 and 0.01 (very significant)

*': p-value between 0.01 and 0.05 (significant)

!': p-value between 0.05 and 0.1 (marginally significant)

': p-value greater than 0.1 (not significant)

Consumer acceptability is central to product success, as it significantly influences purchase intent and consumer loyalty (Lai, Chang, and Chang 2005). In this study, sensory evaluation data (Fig. 35) reveal a clear preference among panelists for **lemon-flavored kefir** across almost all quality parameters, including **odor, taste, acidity, creaminess, and overall acceptability**. The only exception was **appearance**, where the control sample received higher scores, likely due to its unaltered, natural kefir appearance which some consumers might associate with authenticity or freshness.

The **textural properties** of flavored kefir were particularly well-received, with panelists describing it as **thicker, creamier, and smoother** than the control. These qualities may be attributed to the flavoring process, which could influence kefir's structural attributes, enhancing its mouthfeel and sensory appeal. Additionally, the flavored kefir was noted for its **stronger acidic fragrance**—a profile commonly favored in fermented dairy products.

As stated by Dinkci et al. (2015), **taste and consistency** are paramount in determining overall consumer acceptance. Although their findings indicated that control samples were generally rated higher, the addition of lemon flavoring in this study altered the sensory balance, elevating

the acceptability of flavored kefir among panelists. This difference underscores the significant role of **flavor and texture** modifications in shaping sensory preferences.

Supporting these results, Arroum et al. (2023) emphasized that **acidity, flavor, taste, and odor** are highly sensitive to dose and fermentation time, each contributing meaningfully to consumer perceptions of kefir's acceptability. In the case of goat's milk kefir, flavoring can be especially beneficial; according to Tratnik et al. (2006), fermenting goat's milk with kefir grains can help **mask the characteristic “goaty” flavor** that some consumers find off-putting, making the product more palatable. This study's findings align with these observations, as the lemon-flavored kefir was perceived as more balanced in taste and odor, likely due to the masking effects of lemon extract.

Moreover, **kefir grains affect sensory quality** by shaping texture and consistency, which influence creaminess and smoothness (X. Shi et al. 2018). Panelists in this study reported the highest acceptability for the lemon-flavored kefir on **Day 4 of storage**, a timeframe that corresponds to peak freshness for many fermented dairy products. Consistent with findings from Yilmaz, Özcan Yilsay, and Akpinar Bayizit (2006), flavored kefir samples maintained acceptable sensory characteristics throughout the storage period, indicating stability in flavor and texture. In summary, the sensory analysis demonstrates that **lemon-flavored kefir holds a clear advantage** in consumer acceptability, with high ratings for key attributes such as odor, taste, acidity, and texture. These results highlight the potential of **natural flavoring agents like lemon extract** to enhance sensory qualities and broaden kefir's consumer appeal. This insight is valuable for product development, suggesting that strategic flavor modifications can elevate goat's milk kefir's marketability, making it more attractive to a diverse range of consumers.

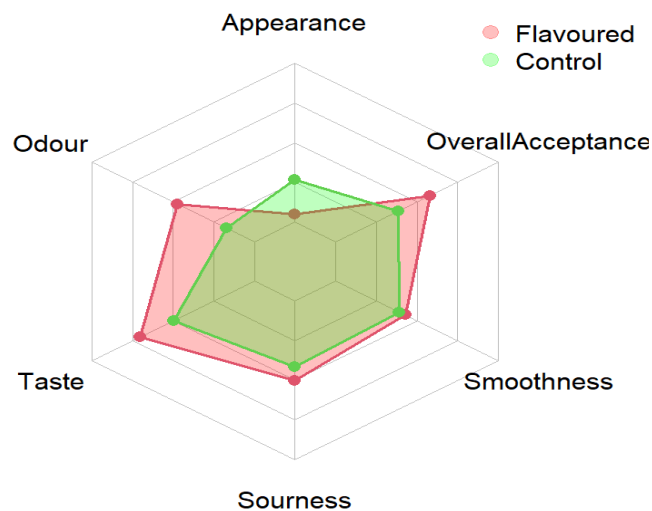


Figure 24: Spider web plot of the respondents' appreciation of kefir

V. Conclusion Remarks and Future Perspective

Fermented milks have a rich history dating back centuries; however, it was only in the late 19th century that their benefits were formally recognized to extend beyond shelf life and sensory appeal to include potential health advantages. This study aimed to verify the relationship between the shelf life of kefir and its evolving physicochemical and microbiological composition under varying conditions, highlighting factors that impact kefir's quality over time.

The first phase of this study was instrumental in defining the optimal parameters for kefir production. Through careful analysis, we identified a specific ratio, temperature, and incubation time to produce kefir with desirable characteristics. This combination allowed us to meet targeted physicochemical criteria (low syneresis, low pH, high acidity), textural qualities (high firmness, strong consistency and cohesion, low viscosity), and microbiological standard benchmarks (sufficient levels of lactic acid and mesophilic bacteria). The results suggest that using a ratio of 0.9%, a temperature of 20°C, and an incubation time of 24 hours is ideal for producing a stable and desirable kefir made of pasteurized goat milk. This optimized combination served as a basis for further research to enhance kefir's shelf life.

The second phase demonstrated significant differences in the evolution of flavored and control kefir over time, notably in their physicochemical, textural, microbiological, and sensory properties. The flavored kefir maintained a lower pH, likely due to lactic acid bacteria activity, which benefits pathogen inhibition (optimal at pH 4.2–4.6). Although syneresis increased in both kefir types, proteolysis was less pronounced in flavored kefir, which contributed to its textural stability. While both types reached similar firmness and cohesiveness, flavored kefir distinguished itself with a higher viscosity index and consistency, attributes that positively affect mouthfeel and consumer preference. In terms of microbiological stability, both kefir types showed comparable composition on the last storage day, with the control kefir having a slight advantage in lactic and mesophilic bacterial levels.

Furthermore, sensory analysis showed a distinct consumer preference for flavored kefir, which was rated superior in odor, taste, acidity, and overall acceptability. This highlights the positive impact of lemon extract flavoring on kefir's sensory attributes and underscores aromatization's influence on kefir's physicochemical, textural, and microbiological properties during storage.

In conclusion, this study provides valuable insights into the composition and storage stability of natural and flavored goat's milk kefir, contributing to a better understanding of how aromatization affects kefir over time.

Future research should expand upon these findings by exploring nutritional and therapeutic properties to further characterize the benefits of goat's milk kefir. Additionally, investigating optimal packaging solutions could enhance product longevity and maintain kefir's sensory quality throughout its shelf life, positioning goat's milk kefir as a highly desirable and health-promoting fermented dairy beverage.

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